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# AN ENCEPHALITIS-NEPHRITIS SYNDROME IN THE DOG PROBABLY DUE TO CONGENITAL ENCEPHALITOZOON INFECTION

By

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## INTRODUCTION

A Foxhound bitch in a hunt kennels gave birth during 1947 to a litter of six apparently normal puppies. According to the attendant, two died later with symptoms similar to those described below. The others reached maturity and showed no abnormality. The bitch was served, in 1948, by the same dog and whelped six live puppies on 8th August. The kennelman stated that these "staggered" about abnormally from the time they were able to walk, but no precise age at which this symptom was first shown could be ascertained. The litter was examined by a veterinary surgeon on 18th September, at which time they were about six weeks old. No other litters were involved.

## CLINICAL HISTORY

*Symptoms.* Three of the six puppies showed severe symptoms, two were less markedly affected, one was clinically normal. In the severe cases there was some loss of condition, but apart from this all symptoms could be related to the nervous system; there was no pyrexia or other systemic sign. The most noticeable feature was a weakness and inco-ordination of the hind quarters, manifested by swaying of the haunches during progression and a tendency when standing still to sink down suddenly behind. Affected animals tired easily and were apathetic; progression between two points could be satisfactorily accomplished along a straight line, but the body was moved diagonally. Pressure on the loins caused exaggerated "dipping". A peculiar spasmodic movement of the eyes, in which both were simultaneously drawn upwards and outwards, was regarded as characteristic. There was no apparent interference with vision.

*Treatment.* No precise diagnosis being reached, two animals were given penicillin, two sulphapyridine and two both drugs simultaneously. Slight temporary improvement only was reported in the sick animals.

*Subsequent History.* Two animals died before 15th October. Post-mortem examination of one was said to have revealed abscesses of the spleen; the other, according to the kennelman, had an enlarged spleen and liver. At this date three others were out "at walk", one of which showed mild symptoms. One (68 days old)

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was destroyed by intravenous nembutal and brought for post-mortem examination. Two others developed symptoms which became increasingly severe. No. II (12 weeks old) was destroyed and examined on 1st November and No. III (seven months old) on 22nd February. On 31st October, the sixth puppy died after having shown eye symptoms and anorexia for some time. It was not autopsied.

The bitch was destroyed before a diagnosis could be reached, and no examination was carried out.

### POST-MORTEM FINDINGS

*No. I.* The carcase was in poor condition, relative to that of the litter-mates. The only abnormality noted was in the kidneys, which were cut with somewhat greater difficulty than normally. The surface was, however, smooth and the capsule detached freely; on incision the cortex appeared to be slightly pale. Macroscopically the brain was normal.

*No. II.* Numerous pin-head sized haemorrhages were noted in the gastric mucosa and a patchy congestion of the duodenum. Both kidneys had an irregular surface, the capsule stripping with difficulty to leave a pitted appearance. The brain and spinal cord showed no macroscopic abnormality.

*No. III.* A few irregular fibrous streaks were found on the capsule of the liver, especially towards the edges of the lobes. The splenic corpuscles were prominent. Small raised nodular lesions up to 1 to 2 mm. in diameter were found on the upper surface of the epiglottis. In the pleural cavity there was an excess of a slightly blood-stained fluid. The diaphragmatic lobe of the left lung was the seat of a fibrinous pleurisy with no adhesions and on incision this and the other lobes showed a severe congestion.

The peritoneum overlying the sub-lumbar region was thickened and coursed by numerous large blood vessels including very prominent veins which appeared to join the spermatic veins posteriorly and disappear anteriorly in a plexus towards the posterior poles of the kidneys. The peritoneum was widely adherent to the renal capsule on both sides and on stripping it away, the kidney surface exhibited a honey-comb appearance due to its subdivision into polygonal areas, of approximately 2 to 5 mm. in size and formed by the development of fibrous bands between the capsule and the cortex. The centre of each area of cortex thus delimited was sunken and often retracted from the overlying capsule, leaving an empty space after fixation. The extirpated kidney appeared to be larger than expected in a dog of this size and the perirenal tissues showed a gelatinous infiltration. On sagittal incision, cortex and medulla maintained about the usual relationship in thickness; the former was streaked by radial bands of fibrous tissue, which at the surface formed the polygonal markings noted above. The medulla was firm, homogeneous and pale.

The submaxillary and pharyngeal lymphatic glands were enlarged and firm as also were the sublumbar and renal glands. The latter on incision presented some degree of congestion whilst the former were pale and fleshy.

No macroscopic abnormalities of the brain or meninges were noted.

HISTOLOGICAL FINDINGS

Material from organs macroscopically abnormal together with the brain and (in No. II) the spinal cord were fixed in formol-saline, embedded in paraffin, sectioned and stained by haematoxylin and eosin. Kidney and some other tissues were also stained by Masson's trichrome method for connective tissue and by Foot's method for reticulin. Some sections of the brain were stained for myelin by Loyez method, and for nerve cells by Giemsa's or Leishman's stain. These two, together with Wright and Craighead's method (carbol-fuchsin-methylene blue), especially the latter, were useful for demonstrating the parasite later to be described.

*Puppy No. I*

The kidney showed diffuse interstitial cellular accumulations extending from the surface almost to the renal pelvis; these were most marked in the deeper layers of the cortex and the outer medulla, especially the latter. The cells comprising these infiltrations were principally plasma cells, endothelioid cells and lymphocytes. The first two occurred usually in rather compact masses between the tubules. The plasma cells were of a typical appearance as described by Marschalko, while the endothelioid cells had an abundant rather eosinophilic cytoplasm and a rounded or oval, somewhat leptochromatic, nucleus, usually with several prominent nucleoli. In the medullary region, especially, there were also scattered foci in which macrophages and a few polymorphonuclears were in process of removing sections of degenerating tubular epithelium. Such areas had a less compact arrangement. Where large interstitial cellular accumulations had formed, the centre was often composed largely of endothelioid elements, with plasma cells and lymphocytes predominating peripherally. In the cortex a noticeable feature was the tendency for limitation of the infiltration to the medullary rays, with relative sparing of the convoluted tubules and glomeruli. A few Langhan's-type giant cells were found, but no large area of necrosis and no calcification; some tubules contained small hyaline, granular or cellular casts. Reticular fibres were increased along the medullary rays. The general picture was thus one of a subacute tubular and interstitial nephritis, particularly severe in the outer medullary and boundary zones and medullary rays.

The liver was severely congested. There were a few small scattered foci, usually rounded, and composed of mononuclear cells of a histiocytic type. They appeared to be more frequent near

vessels of the portal tract and to compress the surrounding parenchyma. Sometimes their cells contained granules of a brownish pigment.

In the brain there was a generalised thickening and cellular infiltration of the meninges with occasional granulomata. The cells concerned in the former were plasma cells and lymphocytes, which were scattered between the fibre bundles of the dura or were lying loose in the interstices of the pia-arachnoid. The appearance of these tissues suggested an oedema. The compact foci or granulomata, afterwards found to be characteristic of the infection and to occur with even greater frequency within the nervous tissue, were composed mainly of endothelioid type cells with an abundant, well-stained, eosinophilic cytoplasm and a large, round, rather lightly-staining nucleus. These granulomata, here as elsewhere, occurred with greater frequency in the neighbourhood of small blood vessels, usually arterioles, which they often surrounded. In several instances it was noted that the muscularis of a small artery was involved; necrotic muscle fibres were in process of being resorbed by polymorphonuclear leucocytes and macrophages. Small arterioles might become completely thrombosed. Larger granulomata were roughly differentiated into an inner zone of endothelioid cells whilst outwardly lymphocytes, plasma cells and an occasional granular leucocyte could be found.

Proceeding inwards at right angles to the meninges were numerous blood vessels with a cuffing of mononuclear cells and histiocytes. These cells seemed to arise from the adventitia of the vessel and not to lie free in the Virchow-Robin space. Histiocytes were usually orientated in the direction of the long axis of the vessel. This type of lesion was most conspicuous in the cerebral cortex.

In the nervous tissue itself two distinct types of lesion were observed. They occurred in both grey and white matter, but were possibly more common in the former, especially in the cerebral and cerebellar cortices. The first type showed changes which in their relationship to blood vessels, and in their cellular content, were identical with those seen in the meninges. In addition, however, this type of lesion was surrounded by a zone of glial proliferation, in which the rod-shaped nuclei of microglia (Stabchenzellen) and macroglia (astrocytes) were prominently increased. Some of the latter were binucleate. The second type of lesion was more diffuse and ill-defined, consisting of an area of mononuclear infiltration and gliosis which usually had no relation to blood vessels, but was seen in a subependymal situation.

In and around both types of lesion occasional nerve cells showed degenerative changes such as chromatolysis, pyknosis karyolysis and eccentric nucleus, but no satellitosis was seen. Myelin preparations failed to show any evidence of demyelination but made possible the detection of the probable causal organism which

appeared to be an encephalitozoon (see page 88). Some displacement, only, of nerve fibres was caused by the granulomata.

*Puppy No. II*

In the kidney the same interstitial cellular infiltration was present as in No. I, again most pronounced in the outer medulla and medullary rays. It was, however, apparent that some fibrosis was commencing, this being particularly obvious in the medulla and around certain groups of the convoluted tubules. There were occasional dense foci of plasma cells and lymphocytes, but in the majority of places the cells were loosely arranged and included many endothelioid elements. The collecting tubules were often contracted from their supporting tissue and their cells shrunken and hyperchromatic. There appeared to be a reduction in the number of tubules in the medulla, their place being taken by the typical fibro-cellular infiltrations. Small hyaline, cellular and granular casts were frequent. There was a distinct tendency to the formation of fibrous streaks along the medullary rays, and, where these emerged at the surface, there was adhesion to the capsule and some retraction. Some glomeruli in and near these fibrotic streaks were densely cellular, contracted and obviously atrophic. No encephalitozoon could be found. In the thoracic spinal cord a cellular infiltration of a ventral nerve root was observed, this being composed of mononuclear and endothelioid cells. Such lesions can obviously give rise to the weakness and incoordination of the limbs noticed clinically. The meninges showed a slight histiocytic and lymphocytic increase whilst the central canal contained granular material with a few macrophages and lymphocytes.

A small cellular focus was observed in a dorsal column, which showed a loose structure, with some compound granular corpuscles. A diffuse nuclear increase in the grey matter, particularly of the ventral columns, was due to glial and mononuclear proliferation. Many cells of the ventral nerve roots were degenerating, leaving vacuole-like spaces. Signs of degeneration, included karyolysis, chromatolysis and disintegration of the cytoplasm were detected, but again no satellitosis. Parasites were found in a small perivascular granuloma.

In the brain there was a definite thickening of the meninges, especially of the cerebrum, where dense collagen bundles were found interspersed with lymphocytes, plasma cells and histiocytes. Some nodular lesions extended across the subarachnoid space into the cortex which showed numerous polymorphonuclears at this point. The same involvement of the muscularis of small arteries as seen in No. 1 was again observed. Granulomata and more diffuse lesions as described above were found in the nervous tissue. A pseudocyst was observed in the endothelium of one such small affected artery.

*Puppy No. III*

In the kidney the most marked feature was a diffuse fibrosis of the medulla with only a few darkly staining, atrophic collecting tubules remaining. Fibrosis was particularly marked around the vessels of the boundary zone extending outwards in streaks between relatively normal areas of the cortex. Some glomeruli, especially within and near the streaks, were contracted and partly or completely hyalinised, with a dilated capsule and concentric collagen bundles around them. Small calcified foci were also contained within the streaks. Two medium-sized arteries were found in the boundary zone which showed complete thrombosis, and it is suggested that their slow occlusion may have caused the great development of a collateral circulation observed at post-mortem examination.

Cellular areas, composed of plasma cells, lymphocytes and a few macrophages were also found interstitially in the medulla and boundary zone, but less frequently than in the two former cases. The great thickening of the capsule with adhesions to the fibrous cortical streaks and to the peritoneum, was well demonstrated in sections stained by Masson's method. No parasites were found in sections stained by the methods of Giemsa, or of Wright and Craighead.

In the larynx small subepithelial nodules were seen which were rather ill-defined and composed largely of small lymphocytes with a few macrophages and a tendency to peripheral fibrosis. These lesions did not resemble those due to encephalitozoon and no parasites were found within them.

The lung showed in affected areas an intense congestion with alveolar haemorrhage. The lumina of the bronchi appeared to be filled by accumulations of macrophages with a few polymorphonuclear leucocytes and lymphocytes. No parasites could be found. The pleura was somewhat thickened and fibrous.

The liver was apparently normal.

The spleen showed congestion of the red pulp and prominent Malpighian corpuscles, the centres of which were composed mainly of small lymphocytes.

In the renal lymph gland a lymphoid hyperplasia of the cortex and congestion and sinus "catarrh" of the medulla were present.

### CAUSAL ORGANISM

The causal organism was first demonstrated in Case I. It was noticed that the myelin preparations of the cerebellum and brain stem, in the first instance, included cyst-like structures with no surrounding tissue reaction. They were composed of large numbers of closely-packed organisms (50 to 100 or more) included in a rounded or oval space delimited by a "membrane" formed by condensation or compression of the surrounding tissues. Individually these organisms were estimated to be about the size of a

corynebacterium (1 to 2 $\mu$); morphologically they were straight or slightly curved bodies staining a deep purple-black, with a well-defined, limiting membrane, round ends and a tendency to polar staining. One end might be narrower and more pointed. Other similar cyst-like structures were next found within the endothelial cells of vessel walls, both normal and those involved in granulomata. They were not detected in neurones whose identity was still recognisable, but were easily found in the endothelial cells of focal lesions. In some situations, especially in the granulomata and their vicinity, organisms were seen lying in small loose clumps or singly, either intra- or extra-cellularly. They were found in areas of degeneration of the muscularis of small arteries and in the same situation in apparently unaltered vessels, thus providing some evidence that they may parasitise smooth muscle cells. The organisms showed the following characteristic staining reactions: —

They were frankly Gram-positive. They stained a bluish-purple by the methods of Leishman or Giemsa. They could be seen, indistinctly, as pale-bluish structures in haematoxylin and eosin sections. The method of Wright and Craighead picked them out beautifully as bright red structures against a blue background. This method also demonstrated the polar staining.

A re-examination of kidney sections stained by haematoxylin and eosin now revealed that the same organisms, in extremely large numbers, were present in the areas of degeneration and infiltration. Pseudocysts were found interstitially, lying free or in endothelioid cells, and, after special staining, could be easily recognised in tubular epithelium and free in the lumen of the tubules. They were most numerous in Henle's loops and the collecting tubules and might occlude them, or, after rupture of the cyst wall, form the granular casts of haematoxylin and eosin sections. Organisms could be found in vascular endothelium and they were seen once in the endothelium of a glomerulus and the outer layer of Bowman's capsule.

It was concluded that these organisms could be no other than Encephalitozoon.

## DISCUSSION

The combination of a nephritis, primarily interstitial and tubular, and a meningo-encephalomyelitis characterised by granuloma formation in relation to small vessels, is at least suggestive of Encephalitozoon infection. In two of three animals it was possible to demonstrate in some of these lesions parasites, the morphology, staining reactions and distribution of which closely corresponded with descriptions of *Encephalitozoon cuniculi*. In this respect we have been greatly aided by reference to the excellent table of comparison of Toxoplasma and Encephalitozoon compiled by Perrin (1943a). The relative ease with which parasites could be found in sections of the kidney stained by haematoxylin and eosin, particularly in case No. I was perhaps an unusual feature.

The nephritis very closely simulates that described by Twort (1922b) and later by Smith and Florence (1925) under the title "Encephalitozoon as a Kidney Parasite". The lesions of the central nervous system are, with the exception that no typical central necrosis was ever observed in the granulomata, identical with those described for the rabbit (Twort, 1922b; Bull, 1917; Oliver, 1922; Wright and Craighead, 1922; Seifried, 1929) as typical of "infectious motor paralysis" or "enzootic encephalomyelitis". Perrin (1943a) described similar lesions in the mouse, rat and guinea pig.

The inability to find Encephalitozoon in the kidney of puppies Nos. II and III recalls a similar observation by Twort (1922b) on the apparent absence, or at least great decrease, of parasites of long-standing kidney lesions in the rabbit. Smith and Florence (1925), in discussing this aspect, state as their opinion, "The invasion is one of the early weeks of life and when the nephritis comes under observation the parasites have been discharged. In the C.N.S., however, such discharge is impossible and the parasites persist intact, since a cellular reaction is absent, at least from many invaded areas". These observations have some significance in the determination of the frequency of lesions due to this organism in the dog population.

In the past there has often been considerable confusion in the differentiation of Encephalitozoon and Toxoplasma. The chronic course and the histopathology of the lesions in these cases differ considerably from any of those described in the literature in cases of canine toxoplasmosis (Mello, 1910; Yakimoff and Kohl-Yakimoff, 1911; Nicolau and Kopciowska, 1935; Machattie, 1938; Ray and Raghavachari, 1941; Olafson and Monlux, 1942; Sjolte, 1948; MacIntyre, Trevan and Montgomerie, 1948; Langham and Sholl, 1949; Wickham and Carne, 1950). The prolonged duration observed by us certainly recalls the symptomless or mild course in many rabbits and other rodents (Perrin, 1943 a and b) with Encephalitozoon infection.

Of great interest is the possibility that congenital infection of the offspring occurred in two successive litters from one bitch. Perrin (1943 b) mentions that the young of artificially infected mice transferred two days after birth to foster-mothers, presumed uninfected, may still develop the condition. It has long been considered that Toxoplasmosis, a closely allied infection is, in many cases, probably congenitally acquired, and a recent series of papers by Cowen and Wolf (1950 a, b, c) has brought confirmation of this suspicion in the case of artificially infected mice. Wickham and Carne (1950) describe two litter-mate Pomeranians which developed Toxoplasmosis, whilst as long ago as 1925 Smith and Florence noted Encephalitozoon infection in successive litters of one doe rabbit.

No examination of the bitch described in this paper was made,

but she was reported to be clinically normal. It is not difficult to speculate on the possible sources of infection, if such did in fact exist. The occurrence of spontaneous Encephalitozoon infection in rodents, including rabbits, rats, mice and guinea pigs, has been recorded by many authors (Bull, 1917; Oliver, 1922; Cowdry and Nicholson, 1924; Wright and Craighead, 1922; Seigfreid, 1937; Perrin, 1943 a). Wild rodents, in contact with dogs, could either be eaten by them, or contaminate their food with urine, an excretion known to contain the parasite in large numbers in some stages of infection. It has been noted in this connection, that Toxoplasmosis can be transmitted by ingestion.

At least two groups of continental authors describe the occurrence of organisms identical morphologically with Encephalitozoon in the brains of dogs thought to have had distemper encephalitis (Kantorowicz and Lewy, 1923; Peters and Yamagiwa, 1936). The two former authors describe cyst-like structures in the nerve or glial cells with some cellular reaction around them; they were also seen in degenerating endothelial cells and thought to resemble *Theileria sp.* more closely than Toxoplasma—the other possibility tendered. They were not ascribed a primary part in the pathogenesis, although present in five of fifteen cases examined pathologically. Peters and Yamagiwa (1936) saw similar parasites in dogs and foxes. They noted that they were found in mixed microglial and " mesodermal " foci distributed as if metastatic, i.e. often in close proximity to vessels, the walls of which showed proliferation of a similar type of cell. In one case they were seen subependymally.

Parasites were also found close to, but outside, these foci and it was stated that they seldom occurred in neurones. Some typical foci had no discoverable parasites, but this also was noted in our cases and may depend on inadequate examination. Without serial sections stained by a selective method it would be difficult to conclude their absence from any particular focus.

Although a confirmation of diagnosis must depend on the isolation of the organism by animal inoculation, it is considered that the probability of this condition being a primary Encephalitozoon infection has been established. In any further suspected cases the examination of the urine should prove of great value. The excretion of organisms via the kidneys has been noted in rodents by many of the early authors quoted, and a smear of centrifuged deposit stained by Giemsa's or other suitable method should reveal the presence of the parasite. Such a deposit could also be inoculated into mice intraperitoneally. Wickham and Carne (1950) noted the excretion of Toxoplasma by the renal route in the rabbit. In addition to parasites one would expect proteinuria and cast formation. Twort (1922 b) records the findings of high blood urea in rabbits.

SUMMARY

A condition of three members of a litter of six Foxhound puppies has been described pathologically and clinically. All six died or were destroyed after a similar clinical course. A nephritis and meningo-encephalomyelitis similar to that occurring in rabbits with Encephalitozoon infection was found.

A parasite identical in nearly all respects to that found in the rabbit and other rodents was demonstrated in lesions of two of the three animals.

Previous observations on similar organisms in dogs are reviewed briefly.

ACKNOWLEDGMENTS

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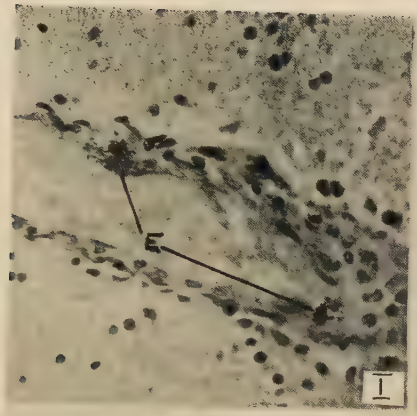
FIG. I. Puppy I. Vessel of medulla oblongata, showing slight cuffing and two accumulations of Encephalitozoon (E) within endothelial cells. (× 600).
FIG. II. Puppy I. Granuloma—cerebral cortex, with parasites scattered singly throughout the central area. × 1,200 approx.
FIG. III. Puppy I. Kidney medulla. Pseudocysts (P) in tubular epithelium and interstitial tissue. (× 600).
FIG. IV. Puppy I. Glomerulus—small group of parasites (E) within endothelial cell. × 1,200.
FIG. V. Puppy I. Kidney medulla—diffuse interstitial infiltration and numerous parasitic pseudocysts appearing very dark (P) + 150.
FIG. VI. Puppy III. Cortex Kidney. Dense fibrous streaking including glomeruli (G). Dilated surviving tubules (left) × 150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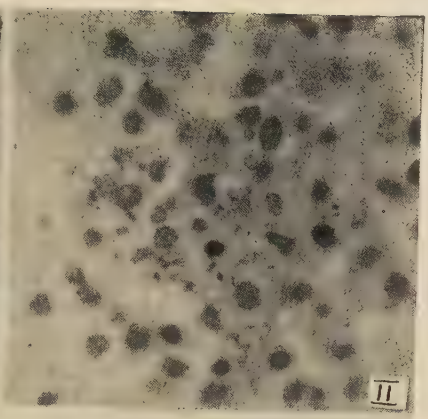
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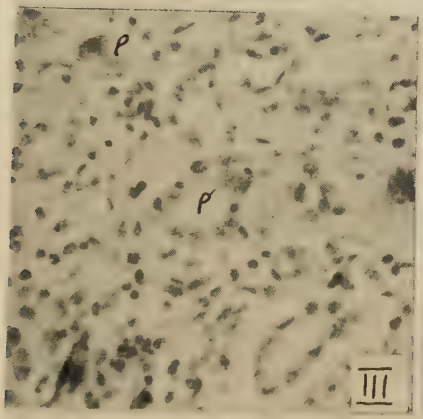
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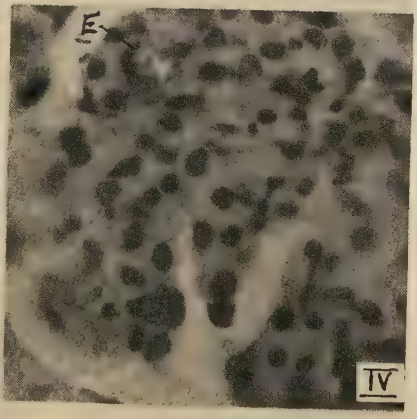
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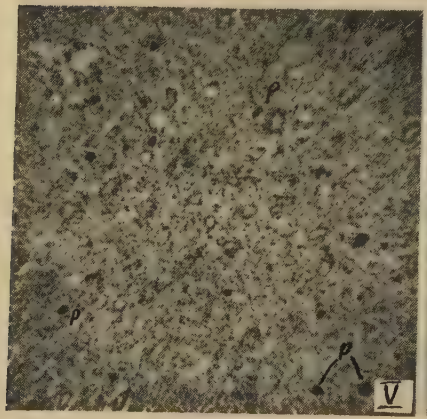
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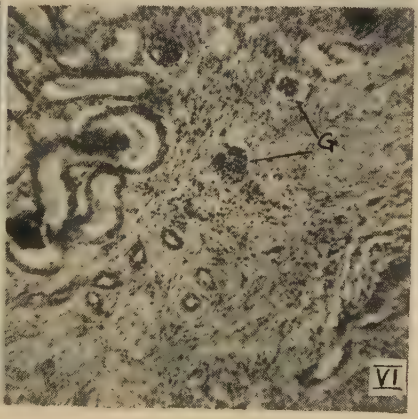
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# ENZOOTIC ABORTION IN EWES

## Complement Fixation Test

By

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### INTRODUCTION

Stamp, McEwen, Watt and Nisbet (1950) described a placentitis of the ewe caused by a virus of the psittacosis lymphogranuloma group (Barwell and Bishop, 1951; Stamp, 1951). The infection gives rise to abortion and premature lambing amongst ewes in the South East of Scotland, a condition which has been prevalent for many years in this area. Although abortion and premature lambing are a feature of the disease, it is known that infected sheep may give birth to normal healthy lambs so that clinical signs are insufficient for assessing the incidence of infection in a flock (McEwen, Stamp and Littlejohn, 1951). It was considered that a laboratory diagnostic test would give more satisfactory information. Since Bedson, Barwell, King and Bishop (1949) had shown the complement fixation test to be accurate in the diagnosis of lymphogranuloma venereum, and Barwell and Bishop (1951) have used the same method for the classification of the present virus into the psittacosis-lymphogranuloma group, it was decided to try to evolve a similar method for field investigation.

### TECHNIQUE

*Egg antigen*

This antigen was prepared in a manner similar to that described by Bedson, *et al* (1949) from the yolk sac of eggs inoculated on the fifth day of incubation and used 5 to 6 days later, only yolk sacs very rich in the organism being used. The growth of the virus in the yolk sac of the developing egg has been previously described by Stamp, *et al* (1950). The yolk sacs were ground in Griffiths tubes and suspended in 2 c.c. of phosphate buffer *p*H 7·6, per yolk sac. After sedimentation in the refrigerator for 24 hours, the supernatant fluid was centrifuged for 2 hours on an angle centrifuge at 5,000 r.p.m., the deposit being suspended in half the original volume of saline. When smears of this suspension, stained by the modified Ziehl-Neelson technique, showed a good virus content, the suspension was steamed for 20 minutes and then sodium azide was added to a concentration of 0·3 per cent. to act as a preservative.

*Foetal membrane-antigen*

During the lambing season of 1951 considerable numbers of infected foetal membranes were sent to the laboratory for diagnosis of enzootic abortion. Some of these membranes were extremely contaminated but a number were received in a fresh state and only

slightly contaminated with bacteria. Microscopical examination of smears made from diseased portions of such membranes showed enormous numbers of virus elementary bodies and it was considered that this material might be made into an antigen more easily than infected yolk sacs. The obviously contaminated portions of the membrane were carefully removed and discarded, while the epithelial surface of the remaining portions was scraped and washed with phosphate buffer. Many of the membranes were thickly encrusted with dry, yellow caseous masses which on microscopical examination were seen to be composed almost entirely of virus elementary bodies and along with the more fluid portions of the membrane were carefully preserved for use. The whole of the collected material was broken down in phosphate buffer made up to 400 c.c. per foetal membrane by shaking with small glass beads for a period of one hour. A preliminary centrifugation was then carried out on a large horizontal centrifuge for 10 minutes at 2,000 r.p.m. to get rid of gross particles. The supernatant fluid was next centrifuged for $1\frac{1}{2}$ hours at 5,000 r.p.m. on an angle centrifuge, the deposit being resuspended in 100 c.c. of saline. If an examination of smears showed that the suspension was rich in virus and relatively free from bacteria, it was treated in the same manner as the egg antigen. Finally all the different batches of membrane antigen were bulked and stored in the refrigerator at 4° C.

*The Complement fixation test*

Preserved guinea pig serum (Burroughs Wellcome) was used throughout, a dose of 2 MHD being calculated by the titration of complement in the presence of the standard amount of antigen. The amount of antigen to be used was determined by finding that dilution of antigen which, when used in 0·1 ml. doses, was not anticomplementary and yet had a high antigenic value. The most satisfactory dilution was found to be one in four since the bulked antigen was anticomplementary and antigen diluted more than one in six lost some of its antigenic value. Dilutions of test sera inactivated at 56° C. for 30 minutes were made from 1 in 2 to 1 in 1024 doubling each time, 0·1 ml. of each dilution being used. Fixation was for 20 minutes at room temperature followed by 30 minutes at 37° C. in the water bath. Overnight fixation in the refrigerator was not satisfactory since the anticomplementary action of the antigen was increased out of all proportion to the increased fixation achieved. The haemolytic system comprised 0·2 ml. of 5 per cent. sheep red cells sensitized with 5 MHD of amboceptor. The quantity of fluid in each tube having been made up to 1 ml. incubation was carried out in the water bath for 30 minutes, readings being taken immediately afterwards. The greatest dilution of serum giving complete fixation of the 2 MHD of complement used throughout the tests was taken as the titre. Suitable controls were included together with pooled sera of known positive and negative titres respectively.

## RESULTS

*Diagnostic value of the complement fixation test*

It was first necessary to determine the complement fixing titres of serum samples taken from sheep known to have aborted their lambs on farms where active infection with the virus of enzootic abortion had recently been demonstrated. In the case of a number of these aborting sheep the virus elementary bodies had been actually demonstrated in the foetal membrances, but in the majority of sheep the evidence of infection was only presumptive, but nevertheless reasonably certain on the grounds of clinical evidence and the known presence of infection in the flock. Serum was used which had been taken six weeks after abortion had occurred. Altogether sera from 146 aborting sheep were examined. In addition, sera from 211 sheep which were born and lived on various farms known definitely to have no history of clinical abortion were also tested to act as control sera. The results are recorded in Table I.

It will be seen that none of the control sheep sera gave a titre as high as $\frac{1}{16}$ whereas 81·3 per cent. of the suspected sera gave titres of $\frac{1}{16}$ or over. It is probable that some of the suspected sera giving titres of less than $\frac{1}{16}$ may in fact be sera from non-infected sheep in that amongst the considerable number of specific abortions, a few may have been caused by agents other than the virus of enzootic abortion. The figure of 81·3 per cent. is, therefore, a conservative estimate of the accuracy of the test. From the present investigation it would appear that a titre of $\frac{1}{16}$ can be taken as being suggestive of infection and titres over $\frac{1}{16}$ as being definite evidence of infection. These tests were duplicated using both egg and membrane antigen and the results in every case were similar. For convenience the membrane antigen was used for the remainder of the work.

*Variation in individual titres during a four month period following abortion*

For the purposes of determining the diagnostic value of the complement fixation test, sera were taken approximately six weeks after abortion had occurred when it was considered that the maximum antibody response would have taken place. In addition, in the majority of cases samples were also taken two weeks and four months after abortion, to follow variations in the titre and to determine the period during which the titre would remain at a high level. It is proposed to continue the investigation of this point for a longer period. It was found that between the first and second bleedings, *i.e.* between two and six weeks after abortion, the serum titres remained steady in forty-two cases (37 per cent.), showed a slight rise or fall in 58 cases (51 per cent.), and changed from a negative to positive reading in 13 cases (12 per cent.). In no case did a positive titre become negative during this period. Between

TABLE I

COMPLEMENT FIXATION TITRES OF ABORTING SHEEP ON INFECTED FARMS AND OF HEALTHY SHEEP ON CLEAN FARMS.

| *Number of Sheep* | *Sheep sera giving complete fixation at dilutions of :—* | | | | | | | | | | | | *Positive Complement Fixation* $\frac{1}{16}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | $\frac{1}{2}$ | $\frac{1}{4}$ | $\frac{1}{8}$ | $\frac{1}{16}$ | $\frac{1}{32}$ | $\frac{1}{64}$ | $\frac{1}{128}$ | $\frac{1}{256}$ | $\frac{1}{512}$ | $\frac{1}{1024}$ | $\frac{1}{2048}$ | |
| 146 ewes ... ... | 0 | 2 | 12 | 14 | 30 | 24 | 27 | 21 | 11 | 3 | 2 | 0 | |
| (All having aborted) | (0%) | 1·3% | 8% | 9·4% | 20·1% | 16% | 18% | 14·7% | 8% | 2% | 1·3% | 0% | 81·3% |
| 122 (65 hoggs) (57 ewes) From non infected farms. | 33 | 39 | 39 | 11 | — | — | — | — | — | — | — | — | Nil |
| | 27% | 32% | 32% | 9% | | | | | | | | | |
| 89 (25 hoggs) (64 ewes) From non infected farms. | None gave a titre of $\frac{1}{16}$ — lower titres were not recorded. | | | | | | | | | | | | Nil |

the second and third bleedings, *i.e.* between six weeks and four months after abortion, the titres remained steady in 54 cases (48 per cent.), showed a slight rise or fall in 55 cases (49 per cent.); in no case did a negative reading change to a positive one. In four cases the readings dropped from a suspicious titre of $\frac{1}{16}$ to a negative reading.

The results indicate that immediately after virus abortion, a small number of sheep gave negative complement fixing titres which six weeks later, changed to positive readings. With the exception of four cases which gave readings of $\frac{1}{16}$ at the second test and were negative at the first and third tests, all the positive reactors continued to react for a period of four months after abortion.

*Determination of incidence of infection in various age groups of sheep on known infected farms*

Clinical manifestations of the disease are of very little value in determining the true incidence of infection in a flock since, although abortion is a feature of the disease, it is assumed that lambs may be born at full term when they may be weakly or quite normal and vigorous (McEwen, *et al*, 1951). In consequence, it was considered that the complement fixation test might give useful information on the actual incidence of infection in various age groups. Four farms were selected to carry out this investigation. Farm A was chosen since over a number of years the incidence of the clinical disease had been fairly constant and was representative of the disease as it occurs commonly on the Border farms of S.E. Scotland. Farm B was chosen since there had been a high incidence of abortion in the current lambing season but the disease had not previously been recognised. Farm C had had an average incidence of clinical abortion for many years, but during the period immediately preceding the tests only a few abortions had been observed each year. Farm D appeared to have no abortion history, but for the last two years quite a number of "rotten" lambs had been born.

Owing to lack of facilities it was not possible to test every sheep on these farms, but random selection of about fifty sheep in each group was carried out. The samples were taken about four weeks after lambing time.

The results, given in Table II, are based on the assumption that a complement fixation titre of $\frac{1}{16}$ or over is evidence of previous infection. The complement fixation titres are given in Table III.

The incidence of clinical abortion is low when compared with the incidence of infection as shown by the complement fixation test. This is particularly evident in the gimmers on Farms A and B. It is realised that some of the infection in the gimmers may have developed after lambing time, but even if the percentage infection of the hoggs is taken as an indication of the incidence in

TABLE II

PERCENTAGE OF INFECTED AND ABORTED ANIMALS IN VARIOUS AGE GROUPS ON FARMS A, B, C, AND D.

| | *Farm A* | | *Farm B* | | *Farm C* | | *Farm D* | |
|---|---|---|---|---|---|---|---|---|
| | *Infected* | *Aborted* | *Infected* | *Aborted* | *Infected* | *Aborted* | *Infected* | *Aborted* |
| Lambs... ... | 2% | — | 5% | — | 0% | — | 2% | No abortion but a number of "rotten" lambs born. |
| Hoggs ... ... | 33% | — | 29% | — | 25% | — | 16% | |
| Gimmers ... | 44% | 1·6% | 64% | 0·8% | 20% | nil | 16% | |
| 2nd Crop ... | 50% | 9% | 70% | 9% | 24% | nil | 37% | |
| 3rd Crop ... | 30% | 5·2% | 72% | 13% | 26% | nil | 36% | |
| 4th Crop ... | — | — | 60% | 7% | 16% | nil | — | |

TABLE III

COMPLEMENT FIXATION TEST IN VARIOUS AGE GROUPS OF SHEEP ON INFECTED FARMS.

| | *LAMBS* | | | | | | *Total Number of Animals* |
|---|---|---|---|---|---|---|---|
| | $o-\frac{1}{8}$ | $\frac{1}{8}$* | $\frac{1}{16}$ | $\frac{1}{16}$* | $\frac{1}{32}$ | $\frac{1}{64}$ | |
| Farm A ... | 49 | — | 1 | — | — | — | 50 |
| Farm B ... | 36 | 3 | 2 | — | — | — | 41 |
| Farm C ... | 50 | — | — | — | — | — | 51 |
| Farm D ... | 47 | 2 | — | — | 1 | — | 50 |

| | *GIMMERS* | | | | | | |
|---|---|---|---|---|---|---|---|
| | $o-\frac{1}{8}$ | $\frac{1}{8}$* | $\frac{1}{16}$ | $\frac{1}{16}$* | $\frac{1}{32}$ | $\frac{1}{64}$ | |
| Farm A ... | 19 | 9 | 11 | 6 | 3 | — | 48 |
| Farm B ... | 12 | 6 | 8 | 9 | 11 | 4 | 50 |
| Farm C ... | 31 | 5 | 1 | 8 | — | — | 45 |
| Farm D ... | 35 | 7 | — | 3 | 3 | 2 | 50 |

| | *3rd CROP* | | | | | | |
|---|---|---|---|---|---|---|---|
| | $o-\frac{1}{8}$ | $\frac{1}{8}$* | $\frac{1}{16}$ | $\frac{1}{16}$* | $\frac{1}{32}$ | $\frac{1}{64}$ | |
| Farm A ... | 15 | 15 | 6 | 1 | 5 | 1 | 43 |
| Farm B ... | 6 | 6 | 7 | 9 | 9 | 7 | 44 |
| Farm C ... | 26 | 11 | 2 | 3 | 3 | 5 | 50 |
| Farm D ... | 30 | 2 | 2 | 3 | 12 | 1 | 50 |

| | *HOGGS* | | | | | | *Total Number of Animals* |
|---|---|---|---|---|---|---|---|
| | $o-\frac{1}{8}$ | $\frac{1}{8}$* | $\frac{1}{16}$ | $\frac{1}{16}$* | $\frac{1}{32}$ | $\frac{1}{64}$ | |
| | 23 | 9 | 16 | — | — | — | 48 |
| | 20 | 15 | 10 | 4 | — | — | 49 |
| | 21 | 14 | 5 | 7 | — | — | 47 |
| | 37 | 5 | 2 | 6 | — | — | 51 |

| | *2nd CROP* | | | | | | |
|---|---|---|---|---|---|---|---|
| | $o-\frac{1}{8}$ | $\frac{1}{8}$* | $\frac{1}{16}$ | $\frac{1}{16}$* | $\frac{1}{32}$ | $\frac{1}{64}$ | |
| | 16 | 10 | 6 | 9 | 6 | 5 | 52 |
| | 8 | 7 | 7 | 9 | 12 | 7 | 50 |
| | 21 | 16 | 4 | 4 | 3 | 1 | 49 |
| | 35 | 3 | 5 | 5 | 10 | 3 | 61 |

| | *4th CROP* | | | | | | |
|---|---|---|---|---|---|---|---|
| | $o-\frac{1}{8}$ | $\frac{1}{8}$* | $\frac{1}{16}$ | $\frac{1}{16}$* | $\frac{1}{32}$ | $\frac{1}{64}$ | |
| | No 4th Crop sheep kept | | | | | | |
| | 14 | 6 | 5 | 7 | 13 | 5 | 50 |
| | 36 | 5 | 2 | 4 | 2 | — | 49 |
| | No 4th Crop sheep kept | | | | | | |

* is where complete fixation occurs at the given titre but higher dilutions give partial fixation.

the gimmers before lambing time, the incidence in the gimmer age group is still considerable. On Farms A, B and D the incidence in the 2nd or 3rd crops is considerably higher than in the younger animals. The amount of clinical abortion is also higher, but even in these groups less than one in ten of positive reactors abort. There is also good evidence that a high incidence of infection takes place in young sheep. This may occur within one month of birth; alternatively, hoggs may become infected during lambing time since at this period they are frequently grazing with ewes which have aborted. It is suggested that the very low incidence of clinical abortion in the gimmers, even when the number of positive reactors is considerable, may be due in some way to the relatively high incidence of reactors amongst the hoggs. It may be that sufficient immunity is developed to overcome the majority of infections before the first pregnancy and it is only when infection occurs for the first time at a later age that a higher incidence of clinical abortion occurs. The finding on Farm C support this hypothesis to some extent. Further field survey work is, however, being carried out in an attempt to clarify such problems of epidemiology.

*Lambs reared on ewes which had aborted just previous to becoming foster mothers*

The sera of twelve ewe lambs, suckling ewes which had previously aborted, failed to give positive reactions before four months of age. This finding would suggest that complement fixing antibodies are not passively transferred in the colostrum and that, if infection is transmitted from the aborted ewe to the offspring during suckling, complement fixing antibodies do not develop during this period.

*Sera from other sources*

*Rams.* Sera from fourteen rams which had been used extensively and for a varying number of years on Farm B gave no positive reactions to the complement fixation test.

*Laboratory staff.* Sera from four members of staff who had worked continuously with the virus of ewe abortion over several years (at times perhaps not too carefully) were subjected to the C.F.T. with negative results.

## SUMMARY

The complement fixation test has been used for the diagnosis of enzootic abortion in ewes.

Suitable antigens were prepared both from infected yolk sacs of the developing chick embryo and from infected membranes of the sheep.

The efficiency of the complement fixation test was assessed by testing blood sera from known infected sheep and from presumably non-infected sheep. A titre of $\frac{1}{16}$ would appear to be suggestive of infection and titres over $\frac{1}{16}$ are evidence of infection.

Complement-fixing antibody was present in the majority of cases within two weeks of abortion having taken place and was detectable for at least four months.

The complement fixation test was used to determine the incidence of infection on four farms. There appeared to be a high rate of infection especially in the 2nd and 3rd crop ewes: moreover, lambs and virgin sheep may give a positive reaction.

The sera of fourteen rams were negative.

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# THE ISOLATION AND CULTURAL CHARACTERISTICS OF AN ORGANISM OF THE HAEMOPHILUS GROUP IN CALF PNEUMONIA

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## INTRODUCTION

Since Pfeiffer (1892) first described the influenza bacillus, organisms of this group have been isolated from diseased conditions in man and other animals, sometimes apparently as the primary cause of the disease and commonly in respiratory conditions as a secondary invader, *e.g.* in influenzal pneumonia in the human subject and in swine influenza.

Lewis and Shope (1931) showed that the latter disease was caused by a combination of this organism and a virus and that either factor alone caused a much milder reaction. Lamont (1938), however, working on piglet influenza succeeded in setting up lesions similar to those of the natural disease with *Haemophilus influenzae suis* alone. It is interesting to note that he isolated other organisms from affected animals in outbreaks where the haemophilus was absent, indicating that other secondary invaders frequently took the place of *H. influenzae suis*. Thorp, Shigley and Farrell (1943), working on calf pneumonia, isolated from 18 of 24 cases examined an organism of the Haemophilus group which gave some cross-agglutination reactions with *H. influenzae*. Attempts to reproduce the disease by intranasal, intratracheal and intravenous administration of suspensions of the organism did not give conclusive results and the authors suggested the possibility that a combination of the bacterium with a virus was the real aetiological factor. The lesions described are very similar to those recorded below.

Lamont and Kerr (1939) reported a somewhat similar condition in calves in Northern Ireland. In many cases they isolated a slender gram-negative bacillus which was grown with difficulty on cooked blood agar and died out on subculture. Many of the cultures, however, showed growths of *Corynebacterium pyogenes* and "bipolars" and an absence of the gram-negative bacillus which they concluded was a member of the Haemophilus group. These workers were successful in transmitting the disease by contact and by intranasal instillation of pneumonic lung in healthy calves. They also succeeded in setting up the disease with Seitz filtrates of infective material. They recovered the haemophilus in one case. Baker (1943) described the isolation of a filterable agent from the lungs of calves suffering from pneumonia. The bacteriological findings were, however, insignificant.

Haemophilus organisms occurring in calves do not appear to have been previously described in Great Britain and the purpose of the present paper is to record the isolation of members of this group from outbreaks of pneumonia of an apparently infectious nature in these animals.

FIRST OUTBREAK

In the first outbreak six calves aged 4 to 8 weeks were affected. In its early stages the disease was very similar clinically to husk, with little or no elevation of temperature, a hard dry cough readily provoked by movement and little interference with appetite. After 4 to 5 days, however, the accelerated and laboured breathing indicated lung involvement. The first calf to show symptoms died after an illness of ten days' duration.

*Post-mortem Findings.* Lesions were confined to the thoracic cavity. A slight pleurisy was present but the main lesions were in the lungs which were largely consolidated. The longer standing lesions were present in the anterior lobes, those in the caudal lobes being of more recent origin. Pus exuded in quantity from the cut surface but no abscesses or evidence of localisation were present. A gram-negative cocco-bacillus, isolated from the active pneumonic areas, was a member of the Haemophilus group.

*Histology.* In both outbreaks the histological picture was that of an acute necrotising broncho-pneumonia. In the smaller bronchioles areas of the epithelium were desquamated and the lumina filled with pus. The alveolar outlines were largely lost and a massive polymorphonuclear invasion was present.

*Haemophilus*

*Colony characteristics.* On 5 per cent ox blood agar plates the colonies at the 24th hour were minute, dome-shaped and surrounded by a narrow zone of $\beta$ haemolysis. After 48 hours the colonies were circular, 1 to 2 mm. in diameter, dome-shaped and transparent with a sharply bevelled entire edge. After four days they became umbonate with a slightly undulate bevelled edge. Colonies were at first mucoid in consistency but later became granular and hard to break up.

*Morphology.* The organism was a gram-negative, pleomorphic cocco-bacillus which stained with difficulty. The best results were obtained with dilute carbol-fuchsin for 10 to 15 minutes. Two types of the organism were present usually in different colonies. In the early cultures the commonest was a tiny cocco-bacillus with rounded ends $1{\cdot}5\mu$ by $0{\cdot}3\mu$. The other type showed marked pleomorphism with variations from cocco-bacillary forms to long strands and threads often in tangled masses crossing the microscope field. Either type of colony gave both morphological forms on subculture, but after some four subcultures only the pleomorphic form was grown. This change in morphology is in accordance with the findings of Wollstein (1915) and others with *Haemophilus influenzae.* The organism was non-motile, non-sporing and no capsule was demonstrated.

*Metabolism.* The organism would grow only in media enriched with blood. Ox, sheep, guinea-pig, and rabbit bloods gave consistently good results, but the organism would not grow in media enriched with horse blood. This apparent inhibitory effect of the blood of certain species on the growth of organisms of the Haemophilus group has been described by Krumwiede and Kuttner (1938). Growth was good in broth enriched with blood and the organism remained viable in this medium when kept in the incubator for periods of one month. Best results were obtained

on lysed blood and chocolate agar plates and growth on Levinthal's medium was satisfactory. Growth occurred well under aerobic, microaerophilic and anaerobic conditions, with quickest growth in an atmosphere of 10 per cent $CO_2$.

Growth took place at temperatures between 30° C. and 42° C. with an optimum of 37° C. Both X and V factors (Topley and Wilson) were necessary for growth. The dependence on these factors was demonstrated by autoclaving chocolate agar at 120° C. for 30 minutes to destroy the thermolabile V factor (Davis, 1921). No growth could be obtained on this medium but the addition of spot cultures of a chromogenic staphylococcus to the plate resulted in the growth of satellite colonies of the Haemophilus organism. No growth could be induced on nutrient agar by the addition of spot cultures or filtrates from broth cultures of the staphylococcus.

*Biochemical reactions.* A weak growth was obtained in the usual fermentation media by the addition of Fildes extract. None of the following reagents were fermented: rhamnose, mannite, salicin, inulin, raffinose, maltose, lactose, saccharose, sorbitol, but slight fermentation of dextrose was observed. Nitrates were reduced. Litmus milk remained unchanged. The organism was penicillin-sensitive being inhibited in blood broth by a concentration of 0·4 units penicillin per ml.

*Pathogenicity.* A suspension from plain blood agar slopes was standardised to tube 3 on the Brown scale. Mice received 0·05 ml. intranasally and 0·5 ml. intraperitoneally: the corresponding doses for guinea-pigs and rabbits were 0·5 ml. and 3 ml. The organism proved non-pathogenic when administered by intranasal instillation and intraperitoneal inoculation. Unfortunately conditions did not allow a trial in calves.

*Antigenic structure.* The strains were submitted to Dr. A. B. Rosher of Mile End Hospital who confirmed that the organism belonged to the Haemophilus group and reported that it did not conform serologically with any of the Pittman strains.

## SECOND OUTBREAK

The second outbreak was much more serious. About twenty calves were affected all under ten weeks of age. They were confined in the stalls of a stable in overcrowded conditions and poor ventilation giving ideal conditions for the spread of respiratory infections. The clinical signs commenced with a harsh cough which persisted for several days. Signs of pneumonia then made their appearance and five of the animals died at this stage. Others lingered on for seven to fourteen days, the majority succumbing, although four made a recovery. Their temperature never rose more than a degree above normal.

When first visited, five animals had previously died, two animals were just dead and some twelve others showing symptoms. In both calves post-mortem examination showed an extensive pneumonia involving the apical and cardiac lobes in their entirety as well as a large part of the caudal lobes. Abscesses were present in the longer standing pneumonic areas of the apical lobes, the cardiac lobes showed grey hepatisation and the caudal lobes sharply demarcated

areas of red hepatisation. Pus exuded in considerable quantities from the cut surfaces. Cultures were made from each of these lobes in both calves as described in outbreak 1.

*Corynebacterium pyogenes* was isolated from the abscesses in the apical lobe in one calf, while a coagulase-positive haemolytic staphylococcus was recovered from the other. Cultures from the cardiac and caudal lobes, however, yielded a haemophilic organism, pure in the first calf and associated with a few colonies of staphylococci in the second. The haemophilic organism did not differ from that described above in its characteristics except that it grew rather more readily and was much less pleomorphic.

*Transmission*

Material from the first of these two calves which had been stored at −70° C. was suspended in broth, centrifuged and the supernatant passed through a Gradocol membrane having an average pore diameter of 0·6μ. This filtrate was bacteriologically sterile and 0·05 ml. was administered to mice intranasally under ether anaesthesia. The mice died at four days and post-mortem examination showed an extensive pneumonia. Five passages had been carried out before calves were obtained for transmission experiments, the mice dying at four to five days in each case. The results of the first series of experiments in calves are given in Table I. The suspension of the Haemophilus was obtained from 36 hour growths on 1 per cent. blood agar slopes and standardised to tube 3 on the Brown scale.

Table I

| *Calf No.* | *Inoculum* | *Route* | *Result* |
|---|---|---|---|
| 1 | 5 ml. haemophilus suspension. ... | Intranasal | Negative. |
| 2 | 5 ml. haemophilus suspension + *filtrate* mouse lung | „ | Extensive pneumonia. Death in 10 days. |
| 3 | *Filtrate* mouse lung alone ... | „ | Slight areas of broncho-pneumonia in apical lobes. |
| 4 | 10 ml. broth ... ... ... ... | „ | Negative. |

Unfortunately at this stage the material was destroyed by the bursting of bottles in the deep freeze apparatus so that it was impossible to carry out further work using the filterable agent from the mouse lung. However, attempts were made to set up pneumonia in four further calves with the haemophilus alone. The results obtained are given in Table II. The haemophilic organism was recovered in pure culture from cases 2 and 5. Case 5 showed no clinical signs of illness but the course of the disease in calf 2 closely followed that described for the natural disease. No evidence of spread from the affected calves was seen.

TABLE II

| *Calf No.* | *Inoculum* | *Route* | *Result* |
|---|---|---|---|
| 5 | 5 ml. haemophilus suspension | ... Intranasal | Small pneumonic areas throughout lung substance. |
| 6 | 5 ml. haemophilus suspension | ... Intratracheal | Negative. |
| 7 | 5 ml. haemophilus suspension | ... Intravenous | Negative. |
| 8 | 5 ml. haemophilus suspension | ... Intranasal | Negative. |

*Histology.* Sections from the affected lungs in cases 2 and 5 showed the picture of an acute fibrinous broncho-pneumonia. The lesions were rather less acute than in the naturally occurring cases. Large numbers of histiocytes had infiltrated the tissue and polymorphs were less numerous than in the natural cases.

*Further History*

In the first outbreak described sulphamezathine gave good results, the condition clearing up quickly. No further deaths occurred in this group of calves.

In the second outbreak the remainder of the affected calves lingered up to three weeks. Changes in management and housing appeared to check further spread of the condition, but all the more chronic cases died and three were examined post-mortem. A chronic purulent pneumonia was present in each with multiple abscess formation throughout the lung tissue. No actively progressing lesions were found. *Corynebacterium pyogenes* and haemolytic staphylococci were the most commonly isolated organisms.

## DISCUSSION

There are many references in the literature to pneumonia in calves often of an apparently infectious nature. The diversity of the organisms isolated in such cases is noteworthy and the principal purpose of this article is to record the recovery of an organism hitherto unrecorded in calf pneumonia in Britain and to show that it is capable of causing disease when administered to calves in combination with a filterable agent. It is realised that such successful transmission is far from providing conclusive evidence as to the aetiology of the natural disease, especially as the material used had been passaged several times in mice before it was tried in calves. We have no evidence that the mice used were free from latent infection with a pneumotropic virus except that no disease occurred amongst the stock mice and that pneumonia appeared at the first transmission. In one of the instances in which Haemophilus was administered alone to a calf, mild lesions were produced from which the organism was recovered but no clinical symptoms were apparent.

The highly infectious nature of the disease in the field was not borne out in the laboratory using the haemophilic organism. This would appear to indicate that some other factor was involved to which the Haemophilus was secondary. This supposition receives some support from the findings of other workers who have shown that pneumonic conditions involving organisms of the Haemophilus group are frequently associated with a virus infection. That organisms other than Haemophilus are frequently involved in calf pneumonia does not detract from this possibility. This was shown by Lamont (1938) and by the findings in the second outbreak recorded above where the Haemophilus tended to be replaced by other organisms in the more chronic cases. This fact, taken with the observation that the Haemophilus was inhibited by horse blood, might account for previous failures to recover this organism.

It was realised that the disease bore a resemblance to that encountered in hoose especially in the second outbreak. A careful examination was made for the presence of lungworms and larvae both macroscopically and histologically. No evidence of parasitic infestation was found as was not unexpected since in both cases the calves were housed and fed on liquid food, calf meal and a little hay.

## SUMMARY

The isolation, growth characteristics and biochemical reactions of an organism of the Haemophilus group, isolated from calves affected with pneumonia, is described.

The induction of pneumonia in a calf by nasal instillation of the Haemophilus in combination with a filterable agent from penumonic calf lung after serial passage in mice is discussed.

It is suggested that the evidence points to the presence of a virus aetiology in some outbreaks of calf pneumonia.

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# THE VIABILITY AND POTENCY OF FREEZE-DRIED LAPINISED RINDERPEST VACCINE

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## INTRODUCTION

At the F.A.O. Rinderpest Control Meeting in Nairobi, Cheng and Fischman (1948) described a method of immunisation against rinderpest in China, in which tissues from rabbits infected with the Nakamura strain III of lapinised rinderpest virus were used as a vaccine for the protection of cattle. Following the investigations by Brotherston (1951), freeze-dried lapinised rinderpest vaccine, produced at Kabete, is now being employed extensively in East Africa. In Uganda and the Sudan it is used to immunise indigenous cattle, whereas in Kenya, it is restricted to pure-bred European cattle and to grade crosses of European with Zebu cattle. As there was little recorded data on the viability and potency of this product, instructions for handling the virus in the field were drawn up on the assumption that it was very fragile. For example, the vaccine had to be kept on ice and discarded after a short expiry time; after reconstitution, the fluid vaccine had to be used within thirty minutes. The experiments recorded in this paper, were undertaken to determine the activity of freeze-dried lapinised vaccine when tested for viability in rabbits and for potency in cattle under conditions approaching those found in the field in East Africa, so that more definite advice could be given to the field staff using the vaccine.

Peng, Shu and Cheng (1948) quoted by Cheng and Fischman (1948) recorded that lyophylised vaccine, packed in vacuum, maintained its potency at 4° C. (39·2° F.) for at least 105 days; after reconstitution with distilled water directly after desiccation, it was still viable after 2 days' exposure at 22° C. According to Hudson and Wongsongarn (1950) pig-passaged lapinised virus stored at minus 20° C. for 6 days protected buffaloes at dose of 0·001 g. and in one case at dose of 0·0001 g.; after storage for 7 days at room temperature which varied between 27° C. and 30° C. the virus protected cattle at dose of 0·01 g., but after 14 days' storage at these temperatures, only one of two animals was protected at dose of 0·01 g. Brotherston (1951) noted that 0·1 g. of lapinised vaccine produced the typical syndrome in rabbits after storage *in vacuo* for 25 months at minus 20° C. to minus 30° C. and also after 70 days' storage on the bench at 24° C.

VIABILITY IN THE RABBIT

Experiments were undertaken to test the viability of the dried vaccine after storage *in vacuo* at 37° C. and also on the bench at the ambient temperature, which fluctuated from 18° C. to 25° C. Other experiments were carried out to determine the viability of the reconstituted vaccine kept at 37° C. and at ambient temperature.

Titrations were carried out in four- to five-months-old rabbits which were bred at Kabete and each animal received a stated amount of the vaccine injected intravenously in 1 ml. of sterile normal saline. When assessing whether rabbits were infected or not, the thermal reactions were recorded. The animals were killed between 65 and 110 hours P.I. and examined for the typical lesions of rinderpest as described by Cheng and Fischman (1948) and Brotherston (1951). A thermal reaction without accompanying lesions was not regarded as evidence of infection, while a slight or delayed thermal reaction with typical lesions at *post-mortem* was regarded as proof of the viability of the virus.

In experiments I and II the vaccine used was K9 batch 33 (784th rabbit passage of the Nakamura Strain III) which was prepared on 29th September 1950 and stored at minus 20° C. to minus 30° C. for 6 weeks before the commencement of the experiments. This vaccine had a residual moisture content of 1·36 per cent and it was proved potent in that 0·0002 g. immunised cattle. For experiments III and IV other batches of vaccine were used, all of which had, however, immunised cattle at 0·0002 g. The dosage used in experiments III and IV was 1 ml. of 1 : 20 dilution, or 0·05 g. which was half the quantity usually given to infect rabbits in the laboratory.

*Viability of the vaccine stored under vacuum at* 37° C.

Ampoules of vaccine K9 batch 33 under vacuum were stored at minus 20° C. to minus 30° C. for 6 weeks. They were then kept at 37° C. and at intervals, one ampoule was removed and titrated in rabbits. Before the commencement of the experiment the vaccine infected rabbits in quantities of up to 0·000001 g. and after 168 hours at 37° C., the titre had dropped to 0·00001 g. It was noted that the vaccine provoked a delayed thermal reaction in rabbits when the smaller quantities were injected and that this delay was pronounced after the vaccine had been kept at 37° C., although the typical lesions were still present. After 18 and 24 hours' storage, the 0·0001 g. and 0·00001 g. quantities respectively failed to provoke a reaction or lesions and this was attributed to faulty technique. The virus was still present in 0·00001 g. after 168 hours' storage and as all the ampoules had been used by this time, this series of observations had to be concluded. The results are presented in Table I.

TABLE I

| *Storage time hours* | *Uninoc. Control* | *Dose in g.* 0·001 | 0·0001 | 0·00001 | 0·000002 | 0·000001 |
|---|---|---|---|---|---|---|
| Nil | — | o | +++ | +++ | ++ | ++ |
| 6 | — | o | +++ | +++ | ++ | ++ |
| 12 | — | o | +++ | +++ | ++ | * |
| 18 | — | o | — | ++ | ++ | ++ |
| 24 | — | o | ++ | — | ++ | ++ |
| 48 | — | +++ | ++ | — | — | o |
| 60 | — | o | ++ | ++ | ++ | — |
| 72 | — | ++ | ++ | — | — | o |
| 96 | — | ++ | ++ | ++ | ++ | o |
| 168 | — | ++ | ++ | ++ | — | o |

+++ = typical thermal reaction and typical lesions.
++ = delayed thermal reaction but typical lesions present.
— = no thermal reaction and no lesions present.
o = no titration carried out.
* = died from injury.

*Viability of the vaccine stored under vacuum at* 18° C. *to* 25° C.

The same vaccine (K9 batch 33) was titrated in rabbits after storage under vacuum on the bench out of direct sunlight; 0·001 g. of vaccine still proved viable after 120 days but these observations could not be extended as the material was all used. The details are shown in Table II.

TABLE II

| *Storage time days* | *Uninoc. Control* | *Dose in g.* 0·01 | 0·001 | 0.0001 | 0·00001 | 0·000002 | 0·000001 |
|---|---|---|---|---|---|---|---|
| Nil | — | o | o | +++ | +++ | ++ | ++ |
| 10 | — | o | o | ++ | +++ | +++ | ++ |
| 20 | — | o | o | +++ | + | + | ++ |
| 30 | — | o | o | ++ | ++ | — | — |
| 40 | — | o | ++ | ++ | ++ | — | o |
| 50 | — | o | ++ | ++ | ++ | — | o |
| 60 | — | o | +++ | ++ | — | ++ | o |
| 70 | — | o | ++ | — | — | — | o |
| 80 | — | +++ | +++ | ++ | ++ | o | o |
| 90 | — | ++ | ++ | — | — | o | o |
| 100 | — | +++ | ++ | — | o | o | o |
| 120 | — | +++ | ++ | + | o | o | o |

+++ = typical thermal reaction and typical lesions present.
++ = delayed thermal reaction but typical lesions present.
+ = no thermal reaction but typical lesions present : these two rabbits were very young.
— = no thermal reaction and no lesions.
o = no titrations carried out.

*Viability of the reconstituted vaccine kept at* 37° C.

Ampoules taken at random from different batches of vaccine were used. The dried vaccine was reconstituted with normal saline to make a 1 : 20 dilution and then maintained at 37° C. A typical reaction was evoked after 7 hours' storage when 0·05 g. was injected into a rabbit. After 10 to 15 hours at 37° C. the vaccine produced delayed thermal reactions in the rabbits and the lesions were less pronounced; after 17 hours the vaccine caused no reactions at all and the virus was then considered to be inactive. These results are shown in Table III.

TABLE III

| *Storage time hours* | *Injection of Virus* | |
|---|---|---|
| | *Batch No.* | *Reaction to dose of 0·05 g.* |
| 1 | K8–7 | +++ |
| 2 | K8–7 | +++ |
| 3 | K9–55 | * |
| 4 | K9–55 | +++ |
| 5 | K9–59 | +++ |
| 6 | K9–59 | +++ |
| 7 | K9–59 | +++ |
| 10 | K9–56 | ++ |
| 11 | K9–56 | ++ |
| 12 | K9–55 | + |
| 13 | K9–55 | + |
| 14 | K9–61 | ++ |
| 15 | K9–61 | ++ |
| 17 | K9–55 | – |
| 18 | K9–55 | – |
| 24 | K9–56 | – |

+++ = typical thermal reaction and typical lesions present.
++ = delayed thermal reaction but typical lesions present.
+ = delayed thermal reactions and poor lesions present.
– = no reaction and no lesions.
* = died from shock immediately after inoculation.

*Viability of reconstituted vaccine kept at* 18° C. *to* 25° C.

Ampoules from different batches were used and the dried vaccine was reconstituted to 1 : 20 with normal saline. The reconstituted vaccine was kept on the bench out of direct sunlight. After 42 hours at room temperature, 0·05 g. of the material produced the typical lesions in the rabbit, but after 48 hours, no reactions or lesions were detected in the inoculated rabbits. The details are shown in Table IV.

TABLE IV

| *Storage time hours* | *Injection of Virus* | |
|---|---|---|
| | *Batch No.* | *Reaction to dose of 0·05 g.* |
| 1 | K9–54 | +++ |
| 2 | K9–53 | +++ |
| 3 | K9–53 | +++ |
| 4 | K9–53 | +++ |
| 5 | K9–53 | +++ |
| 30 | K9–59 | +++ |
| 31 | K9–59 | +++ |
| 36 | K9–59 | * |
| 36 | K9–59 | +++ |
| 42 | K9–56 | * |
| 42 | K8–7 | ++ |
| 48 | K9–56 | – |
| 48 | K9–55 | – |
| 72 | K9–56 | – |
| 72 | K9–55 | – |
| 96 | K9–56 | – |

+++ = typical thermal reaction and typical lesions present.
++ = delayed thermal reaction but typical lesions present.
– = no reaction and no lesions present.
* = died from shock immediately after inoculation.

POTENCY OF LAPINISED VACCINE AS TESTED IN GRADE CATTLE

Four series of experiments were carried out. The vaccine used was K9 batch 60 (784th rabbit passage) which was prepared on 16th March 1951 and stored at minus 20° C. to minus 30° C. for 5 to 7 weeks prior to these experiments. The residual moisture content of samples of vaccine was found to be 0·58 per cent and material from this batch had been proved potent in cattle in doses up to 0·0002 g. All the cattle used in the experiment were susceptible grade stock purchased from European farmers in the Kenya Highlands. Each animal was inoculated subcutaneously with 2 ml. of a $10^{-3}$ dilution so that each received 0·002 g. of the vaccine under test. This amount of vaccine material was half that normally used in the field, so that the facts obtained could be safely applied to field conditions. The cattle were challenged by the subcutaneous injection of stock Kabete "O" strain virulent bovine rinderpest virus 10 to 14 days P.I.

*Potency of the vaccine stored under vacuum at* 36·8° C. *to* 37·8° C.

Ampoules of K9 batch 60 were stored at 36·8° C. to 37·8° C. and tested after 36, 48, 60, 96 and 132 hours. Prior to each inoculation, one ampoule was reconstituted to $10^{-3}$ with normal saline and 2 ml. inoculated into 2 or more cattle. Vaccine stored for 36 and 48 hours immunised grade cattle against a challenge dose of virulent bovine virus but after 60 and 96 hours the inoculated test cattle were not regularly immunised; after 132 hours, the vaccine

failed to immunise cattle. In view of these results the end point was taken as 48 hours after the reconstitution of the vaccine. (Table V).

TABLE V

| *Cattle Number* | *Storage Time, Hours* | *Dose of Vaccine* | *Result of Challenge* |
|---|---|---|---|
| 1815 | 36 | 0.002 g. | Immune |
| 1822 | 36 | 0·002 g. | Immune |
| 1926 | 36 | 0·002 g. | Immune |
| 1927 | 36 | 0·002 g. | Immune |
| 1810 | — | Control | Susceptible |
| 1937 | 48 | 0·002 g. | Immune |
| 1938 | 48 | 0·002 g. | Immune |
| 1819 | — | Control | Susceptible |
| 1814 | 60 | 0·002 g. | Immune |
| 1802 | 60 | 0·002 g. | Susceptible |
| 1805 | 96 | 0·002 g. | Immune |
| 1804 | 96 | 0·002 g. | Susceptible |
| 1809 | 132 | 0·002 g. | Susceptible |
| 1811 | 132 | 0·002 g. | Susceptible |
| 1932 | — | Control | Susceptible |

*Potency of the vaccine after storage under vacuum at* 18° C. *to* 22° C.

Ampoules of vaccine were stored on the bench, out of direct sunlight and tested after 20, 40, and 60 days. Cattle injected with 2 ml. of vaccine, reconstituted as already described, were protected after 20 and 40 days' storage, but not after 60 days, as shown in Table VI.

TABLE VI

| *Cattle Number* | *Storage Time, days* | *Dose of Vaccine* | *Result of Challenge* |
|---|---|---|---|
| 1807 | 20 | 0·002 g. | Immune |
| 1812 | 20 | 0·002 g. | Immune |
| 1798 | — | Control | Susceptible |
| 1887 | 40 | 0·002 g. | Immune |
| 1886 | 40 | 0·002 g. | Immune |
| 1888 | — | Control | Susceptible |
| 1954 | 60 | 0·002 g. | Susceptible |
| 1964 | 60 | 0·002 g. | Susceptible |
| 1967 | — | Control | Susceptible |

*Potency of the reconstituted vaccine kept at* 37·0° C. *to* 37·8° C.

Vaccine K9 batch 60 was reconstituted with normal saline to make a dilution of $10^{-3}$. The reconstituted vaccine was heated in a waterbath for $\frac{1}{2}$, 1, 2, 4, 6, and 10 hours respectively and 0·002 g. was inoculated into two cattle. On challenge it was found that the reconstituted vaccine kept successfully for half and 1 hour but not for longer periods. (Table VII).

TABLE VII

| *Cattle Number* | *Storage Time, Hours* | *Dose of Vaccine* | *Result of Challenge* |
|---|---|---|---|
| 2030 | $\frac{1}{2}$ | 0·002 g. | Immune |
| 2032 | $\frac{1}{2}$ | 0·002 g. | Immune |
| 2029 | 1 | 0·002 g. | Immune |
| 2031 | 1 | 0·002 g. | Immune |
| 2027 | 2 | 0·002 g. | Susceptible |
| 2028 | 2 | 0·002 g. | Susceptible |
| 1733 | 4 | 0·002 g. | Susceptible |
| 1750 | 4 | 0·002 g. | Susceptible |
| 1736* | 6 | 0·002 g. | Immune |
| 1743 | 6 | 0·002 g. | Susceptible |
| 1744 | 10 | 0·002 g. | Susceptible |
| 1746 | 10 | 0·002 g. | Susceptible |
| 1738 | — | Control | Susceptible |
| 2033 | — | Control | Susceptible |
| 2034 | — | Control | Susceptible |

* Animal immune before the experiment ?

*Potency of the reconstituted vaccine kept at* 20° C. *to* 22° C.

The vaccine was reconstituted as described and stored at room temperature, out of direct sunlight, for 6, 12, 24 and 36 hours. Two millilitres (0·002 g.) were inoculated into two cattle at each period and only vaccine stored for 6 hours protected against the challenge dose of virulent bovine rinderpest virus. (Table VIII).

TABLE VIII

| *Cattle Number* | *Storage Time, Hours* | *Dose of Vaccine* | *Result of Challenge* |
|---|---|---|---|
| 1740 | 6 | 0·002 g. | Immune |
| 1741 | 6 | 0·002 g. | Immune |
| 1734 | 12 | 0·002 g. | Susceptible |
| 1748 | 12 | 0·002 g. | Susceptible |
| 1749 | 24 | 0·002 g. | Susceptible |
| 1739 | 24 | 0·002 g. | Susceptible |
| 1732 | 36 | 0·002 g. | Susceptible |
| 1737 | 36 | 0·002 g. | Susceptible |
| 1742 | — | Control | Susceptible |

## DISCUSSION

The experiments on the viability of the virus of freeze-dried lapinised virus were first carried out in rabbits in order to obtain a "yardstick" before embarking on experiments with the more expensive cattle. The routine dose of lapinised virus used to infect rabbits is 0·1 g. in 1 ml. of saline injected intravenously and the routine field dose for cattle is 0·004 g. in 2 ml. saline inoculated subcutaneously. In these experiments half the routine rabbit and cattle doses have been used so that the results could be safely applied to field conditions.

In Kenya lapinised rinderpest vaccine, when inoculated into European and grade cattle causes no reaction in the majority of cases; thus one cannot easily detect whether the lapinised vaccine is viable or not, as one can when using goat-adapted rinderpest virus which produces a definite clinical reaction. It is, therefore, imperative for field officers to follow strictly the instructions regarding the handling of lapinised vaccine and, similarly, it is important that a large and practical safety margin be allowed. With this in mind the results have been assessed strictly and the findings are such that it is still considered that freeze-dried lapinised vaccine should be despatched on ice unless the material can be used within twenty-four hours of issue. It is also suggested that thirty minutes after reconstitution is the upper safety limit for the vaccine and as each ampoule produced at Kabete normally contains fifty to a hundred cattle doses, this should cause little inconvenience.

## SUMMARY

An investigation into the viability and potency of freeze-dried lapinised rinderpest vaccine is recorded.

It is emphasized that because of the absence of a marked thermal reaction in the majority of cattle inoculated with lapinised vaccine, great care is required to ensure that potent material is inoculated.

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# RELATIONSHIPS BETWEEN SULPHAMEZATHINE THERAPY AND THE ACQUISITION OF IMMUNITY TO *EIMERIA TENELLA*

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## INTRODUCTION

The ability of young chickens to develop a specific resistance to reinfection with *Eimeria tenella*, first recognised by Tyzzer (1929), has been confirmed by numerous workers. The ubiquitous distribution of the parasite and the development of a strong and lasting immunity explain why disease attributed to *E. tenella* is substantially confined to young stock. Most modern methods of controlling caecal coccidiosis in the field take advantage of the fact that the disease is rarely, if ever, fatal among chicks which have recovered from a previous mild infection.

The facts relating to the development and duration of this immunity are less well known although the time necessary for the establishment of immunity is comparatively short. Horton-Smith (1949) concluded that resistance to reinfection appears ten days after the development of the schizonts. In 1947, he had suggested that the second generation schizont stimulates the immune response in the host. If this is so, it is apparent that the very earliest stage of the schizont must be the one involved for it had been shown by Horton-Smith and Taylor (1945) that resistance to reinfection is readily acquired even when the development of the second generation schizont is suppressed by drug therapy.

Our observations will show that resistance to reinfection is developing at the 72nd hour, at which time the second generation schizont is in its earliest stage.

## RESULTS

### *The Minimum Period Necessary for the Acquisition of Resistance to Reinfection*

The Rhode Island Red chicks used in this experiment were kept in isolation until inoculated at ten days old and throughout the experimental period were maintained in electrically-heated wire-floored brooders.

The resistance of chicks which had received a sublethal infection of approximately 2,000 sporulated oöcysts was challenged after various intervals with oöcysts sufficiently numerous to cause a heavy mortality among parallel groups of susceptible birds.

Table I compares the rate of mortality in the immunised groups of chicks ($A_0$, $B_0$, $C_0$, $D_0$ and $E_0$) with controls ($A_1$, $B_1$, etc.) which

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TABLE I

CHALLENGE AT VARIOUS INTERVALS AFTER AN INITIAL INFECTION WITH 2,000 SPORULATED OÖCYSTS OF *Eimeria tenella*

| *Group* | *Initial No. of chicks per group* | *Initial Infection* | *Subsequent Infection* | *Mortality following challenge* |
|---|---|---|---|---|
| $A_o$ | 33 | Immunising infection ... ... ... ... | Challenge at 192nd hour after initial infection ... | 18 |
| $A_1$ | 36 | Control (No immunising infection) ... ... | Challenge as $A_o$ ... | 83 |
| $B_o$ | 32 | Immunising infection ... ... ... ... | Challenge at 96th hour... | 22 |
| $B_1$ | 36 | Control (No immunising infection) ... ... | Challenge as $B_o$ ... | 92 |
| $C_o$ | 42 | Immunising infection ... ... ... ... | Challenge at 72nd hour | 50 |
| $C_1$ | 18 | Control (No immunising infection) ... ... | Challenge as $C_o$ ... | 89 |
| $D_o$* | 51 | Immunising infection ... ... ... ... | Challenge at 48th hour... | 67 |
| $D_1$* | 18 | Control (No immunising infection) ... ..... | Challenge as $D_o$... ... | 61 |
| $E_o$ | 36 | Immunising infection ... ... ... ... | Challenge at 24th hour... | 89 |
| $E_1$ | 32 | Control (No immunising infection) ... ... | Challenge as $E_o$ ... | 94 |

* Lower rate of mortality in both experimental and control groups was attributed to using a challenge inoculum of comparatively low virulence.

had not received any initial immunisation. As anticipated, the chicks in group $A_0$, immunised eight days before their resistance was challenged, showed considerable resistance as compared with the susceptible group $A_1$. The degree of resistance was not, however, substantially different from that exhibited when reinfection took place after 96 hours (group $B_0$) while an initial infection only 72 hours before the challenge (group $C_0$) still protected some of the chicks. On the other hand, there was no appreciable resistance among birds which were challenged 48 or 24 hours after the initial infection.

It appeared, therefore, that resistance to reinfection was developing by about the 72nd hour and that at the 96th hour the chicks had acquired as high a degree of resistance as was apparent by the eighth day. (Group $A_0$, Table I).

*The Use of Sulphamezathine for the Prevention of Deaths from Caecal Coccidiosis*

*Low Concentrations.* The acute haemorrhage which causes mortality from caecal coccidiosis on the fifth day following infection results from tissue disruption associated with the mass migration of the second generation merozoites from the mature second generation schizonts. Horton-Smith and Taylor (1945) demonstrated that replacing the drinking water with Sulphamezathine (Sulphadimethylpyrimidine) in a saturated solution (about 0.13 per cent) controlled mortality in chicks which had been heavily infected with *E. tenella*. They showed an inhibition of development of the second generation schizonts, but did not report any effect on earlier developmental stages of the parasite. Moreover, Horton-Smith and Boyland (1946) showed that 0.2 per cent Sodium Sulphamezathine, by suppressing the second generation schizonts, prevented symptoms in almost all chicks when treatment was delayed for 72 hours. Our observations have confirmed that such low concentrations have a pronounced effect on the second generation schizonts but have little evident effect on earlier developmental stages. This selective effect on the second generation schizonts might, moreover, have been inferred from observations that immunity is becoming established by the 72nd hour (Table I) at which time the second generation schizonts are immature. If the early developmental stages were destroyed by Sulphamezathine it can be assumed that immunity would not develop. In fact, the development of immunity in treated chicks is an essential part of the rationale of Sulphamezathine therapy.

*Higher Concentrations.* While the use of Sulphamezathine at low concentrations has no apparent effect on the earlier developmental stages of the parasite, higher concentrations (of the order of 1.0 per cent of the food) may exert a marked effect.

Farr and Wehr (1947) published a careful analysis of the effect on *E. tenella* of 1.0 per cent Sulphamezathine in the mash. There

TABLE II

COMPARING MORTALITY AMONG CHICKS (INOCULATED WITH 50,000 OÖCYSTS OF *Eimeria tenella*) FOLLOWING MEDICATION WITH 1·0 PER CENT SULPHAMEZATHINE FOR SIX DAYS (GROUP A) AND TWO DAYS (GROUP B).

| | *Days after day of Inoculation (= Day 0).* | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0−1 | Day 0 | 0+1 | 0+2 | 0+3 | 0+4 | 0+5 | 0+6 | 0+7 | 0+8 | 0+9 | 0+10 | 0+11 |
| | | | | | | | | | | | *Deaths from Acute Coccidiosis* | | |
| GROUP A (30 Chicks) | 1·0 per cent. Sulphamezathine fed continuously in dry mash ... ... | | | | | | Plain dry mash fed ... | | | | 3 | 18 | Nil |
| GROUP B (28 Chicks) | Plain dry mash fed ... | | 1·0 per cent. Sulphamezathine fed in dry mash ... | | | | Plain dry mash fed ... | | | | Nil | Nil | Nil |

| | *Total Mortality per cent.* |
|---|---|
| GROUP A (30 Chicks) | 70 |
| GROUP B (28 Chicks) | Nil |

was some effect on the first generation schizonts but the first generation merozoites were resistant. As might be expected the effect on the second generation schizonts was dramatic and they and their contained merozoites appeared to be destroyed.

Wehr and Farr (1947) noted that some of their experimental chicks showed haemorrhage after the medicated mash had been discontinued and after an interval considerably greater than the usual five days from the date of infection. It appeared that 1.0 per cent of the drug in the food during the early part of the developmental cycle produced a coccidiostatic effect. This action is confirmed (Table II) and contrasted with the apparently coccidiocidal effect of the drug when used selectively against the second generation schizonts.

A uniform group of 10-day old R.I.R. chicks was divided into two batches, each chick receiving an ordinarily lethal inoculation with the sporulated oöcysts of *E. tenella*. One batch of birds (Group A) was maintained, from the day before inoculation until the fifth morning following inoculation, on a dry mash which contained 1.0 per cent Suphamezathine. The other batch (Group B) received the medicated mash from the third until the fifth morning following the inoculation. One group, therefore, received the drug for six days and the other for two days. A heavy mortality from caecal coccidiosis occurred among those chicks receiving medicated mash for six days, whereas there was no such mortality among those which received drug only for the third and fourth days following infection. (Table II.)

It would seem that at a concentration of 1.0 per cent, Sulphamezathine by inhibiting the development of the second generation schizonts exerts its ordinary therapeutic effect. If, however, the drug is administered during the earlier developmental stages of the parasite, the coccidiostatic effect is only temporary and withdrawal of the drug permits full development of the parasite as in the uncontrolled disease.

### *Dosage Rates at which the Earlier Development Stages of* Eimeria tenella *are Affected*

Different concentrations of Sulphamezathine in dry mash were fed from the day before infection until the fifth morning following infection. At concentrations of 0.25 per cent and 0.375 per cent of the food, the drug exerted a satisfactory therapeutic effect (Table III). Such mortality as occurred was slight in comparison with the heavy mortality in the control group (F). At higher concentrations of the drug (0.50 per cent, 0.75 per cent and 1.0 per cent) death was delayed until the ninth and tenth days after infection and it is noteworthy that the number of birds which died exceeded that in the group (D & E) receiving smaller amounts of drug.

TABLE III

DELAYED MORTALITY FROM CAECAL COCCIDIOSIS FOLLOWING SULPHAMEZATHINE THERAPY

| *Initial Number of Chicks per group* | | *Concentration of Sulphamezathine % in Dry Ration* | *Drug in Ration* | | | *No Drug in Dry Ration* | | |
|---|---|---|---|---|---|---|---|---|
| | | | *Symptoms and Mortality* | | | *Delayed Mortality* | | |
| | | | *Number of Days following the day of Inoculation (= Day 0)* | | | | | |
| | | | 0 + 4 | 0 + 5 | 0 + 6 | 0 + 8 | 0 + 9 | 0 + 10 |
| (A) | 30 | 1·0 | — | — | — | — | Blood | 8 |
| (B) | 31 | 0·75 | — | — | — | Blood | 7 | 2 |
| (C) | 30 | 0·50 | — | — | — | Blood | 4 | 3 |
| (D) | 31 | 0·375 | — | Blood | 1 | — | — | — |
| (E) | 30 | 0·25 | Blood | Blood | 4 | — | — | — |
| (F) | 30 | *No Drug* | Blood | 24 | 1 | — | — | — |

## DISCUSSION

In uncontrolled caecal coccidiosis most deaths occur on the fifth day following infection and it is therefore significant that the delayed deaths following the use of 1.0 per cent Sulphamezathine occurred on the fifth day after withdrawal of the drug. This clearly indicates that the entire life-cycle of the parasite has been interrupted and it must be inferred that even the sporozoites do not commence to develop until the drug is withdrawn.

At drug concentrations of 0.50 per cent and 0.75 per cent mortality was recorded on the fourth as well as the fifth day following the termination of therapy, indicating that the development of the parasite had been inhibited about 24 hours after the time of infection. At concentrations of 0.25 per cent and 0.375 per cent a few deaths occurred on the sixth day after infection, the drug under such circumstances exerting its usual therapeutic effect and the development of the parasite being allowed to proceed almost unhindered up to the appearance of the second generation schizonts.

It is clear that the different developmental stages of *Eimeria tenella* vary in susceptibility to Sulphamezathine, the earliest stages being least susceptible and the later stages (particularly the second generation schizonts) being more susceptible. At ordinary therapeutic levels (about 0.2 per cent Sodium Sulphamezathine in the water or 0.4 per cent Sulphamezathine in the food) only the second generation schizonts are ordinarily affected. At high levels of the drug other stages are affected until at a concentration of 1.0 per cent even the invading sporozoites are inhibited.

In the absence of drug therapy, infection with *Eimeria tenella* is associated with haemorrhage at about the 96th to the 120th hour. If the bird recovers it will show strong resistance to reinfection. In the light of the immunity experiments (Table 1) this resistance to reinfection is quite apparent by the 96th hour so that its full development is contemporaneous with the maturation of the second generation schizonts. The exact mechanism of this resistance is not known but it seems reasonable to believe that it would prove equally effective against each individual developmental stage of the parasite, including the second generation schizonts. If, therefore, the second generation schizonts can temporarily be inhibited, the developing immunity will hinder their further maturation. The bird will actually show resistance to the current infection while developing its normal resistance to reinfection.

It is suggested, therefore, that Sulphamezathine is a temporary inhibitor, the actual control of the lethal second generation schizonts being assured by the same immunological reaction as that which controls repeated infection. It is evident that Sulphamezathine must be used selectively against the second generation

schizonts and that anything which suppresses the earlier developmental stages is likely adversely to affect the therapeutic action of the drug.

*Field implications of the observations.* While Sulphamezathine may be used with great success for the control of caecal coccidiosis, occasional field reports suggest that it may give unsatisfactory results. Some such instances have been brought privately to our notice by Mr. J. D. Blaxland of the Poultry Diseases Department at Weybridge. Outbreaks of caecal coccidiosis in the field, indicated by haemorrhage from a small portion of the birds, had been apparently controlled by the use of Sodium Sulphamezathine in the drinking water for periods of three to five days. Discontinuation of the drug had, however, resulted in mortality about four days later. This clinical picture was similar to that described in our experimental observations and suggests that under field conditions the drug may sometimes exert a temporary coccidiostatic effect on the earlier developmental stages of the parasite with unfortunate sequelae following discontinuation of the drug.

There is little doubt that under ordinary circumstances the usually recommended concentration of about 0.20 per cent of Sulphamezathine in the drinking water is likely to control haemorrhage while allowing the rapid development of resistance. Nevertheless there is not a very wide margin between this concentration and the 0.5 per cent in the food which may mask the selective effect on the second generation schizonts and lead to delayed mortality. Since chicks consume approximately 1 oz. of dry mash for every 2 fluid ozs. of drinking water, it can be assumed that any given concentration of drug in the food will produce an effect on the parasite similar to that produced by half this concentration in the drinking water. Under field conditions there is likely to be considerable variation in the amounts of drug assimilated at different times by individual chicks and a corresponding variation in the concentrations of drug which affect different developmental stages of the parasite. In such circumstances occasional inconsistency in the therapeutic action of the drug may well be expected.

Two criteria are recognised for the proper use of the drug in the field. Firstly, the drug must be used only at the minimum concentration consistent with an inhibition of the second generation schizonts; secondly, as far as possible, therapy should be limited to the latter period of the development of the parasite. In practice, this may best be assured by interrupted therapy, leaving intervening days without treatment in order to provide for the development of resistance.

## SUMMARY

Resistance to reinfection with *Eimeria tenella* is developed by the 96th hour following initial inoculation.

Drug concentrations of 0.25 per cent and 0.375 per cent of

Sulphamezathine in the food have a selective effect on the second generation schizonts thereby controlling mortality from caecal coccidiosis. Drug concentrations of 0.50 per cent to 1.0 per cent interfere with the normal therapeutic effect by inhibiting developmental stages previous to the second generation schizonts.

In view of the well known therapeutic effect of the drug it can be assumed that Sulphamezathine is coccidiostatic to all developmental stages previous to the second generation schizonts to which it is coccidiocidal. Alternatively, it is suggested that a coccidiostatic effect on the second generation schizont is reinforced, at therapeutic levels of the drug, by the naturally developing immunity.

It is concluded that the use of an excessively high concentration of the drug is not only unnecessary but may produce untoward results as instanced by the occasional failure of Sulphamezathine treatment under field conditions. The therapy must be confined, as far as possible, to the later stages of the development of the parasite.

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# FREEZE-DRYING OF THE ORGANISM OF CONTAGIOUS BOVINE PLEURO-PNEUMONIA

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## INTRODUCTION

Employing a comparatively crude technique, in which a hand-operated Gerrick pump was used, it has already been shown (Priestley, Mihimeid and Dafaalla, 1948) that the contagious bovine pleuro-pneumonia organism can be successfully maintained in a viable state when dried *in vacuo* over phosphorus pentoxide. In the original paper it was noted that cultures had been so maintained for 154 days; it is worth while recording now that, of a batch dried at that time, the latest tube to be opened and grown was dried 1076 days previously.

The acquisition of an Edwards' & Co. centrifugal freeze-dryer (Model L5) made it possible to carry out many more experiments since, with this machine, it is possible to dry off 30 tubes of material in one operation.

The objects of the work recorded here were, firstly, to standardise the seed material for vaccine production, i.e. to dry off the organism at some desired level of attenuation and, secondly, to produce actual dried vaccine. The desirability for standardising the seed material needs no emphasis while the advantage of a dried vaccine product becomes evident when it is realised that the effective "life" of the present serum broth vaccine is only about three weeks from the date of issue (Bennett, 1932).

## VIABILITY COUNTS

It was first necessary to obtain details of the survival time of the organism in serum broth. Accordingly a number of bottles containing 50 c.c. serum broth were sown with 0·5 c.c. of a young culture of the vaccine strain then in use. These bottles were incubated at 37° C. for two days, corked with rubber bungs, sealed with paraffin and incubated for a further two days. Some were then left in the incubator at 37° C. while others were placed in the ice-chest (at about 5° C.). Counts were made from time to time with the results shown in Table I.

Since the pleuro-pneumonia organism grows so poorly and so erratically on solid media it was necessary to resort to dilution counts. One c.c. of the culture to be counted was mixed with 9 c.c. serum broth (8 c.c. broth plus 1 c.c. serum) and serial 1 in 10 dilutions made from this into further tubes of 9 c.c. serum broth. A separate pipette was used for each dilution. All tubes were incubated at 37° C. and the day was noted on which growth occurred. Tubes showing no growth were incubated for at least 10 days before being discarded.

Growths were always confirmed microscopically, using a modified stevenel blue technique (Priestley and Mehdi, 1944); briefly this consists

TABLE I.

THE RESULTS OF VIABILITY COUNTS OF THE PLEURO-PNEUMONIA ORGANISM IN SERUM BROTH.

| *Days stored* | *Highest dilution showing growth after storage in—* | |
|---|---|---|
| | *Incubator (at 37°C)* | *Ice-chest (at 5°C)* |
| 1 | $10^{10}$ | · |
| 2 | $10^{12}$ | · |
| 3 | $10^{10}$ | · |
| 4 | $10^{11}$ | · |
| 5 | $10^{9}$ | · |
| 6 | $10^{9}$ | · |
| 7 | $10^{8}$ | · |
| 8 | $10^{8}$ | · |
| 9 | $10^{7}$ | · |
| 10 | $10^{7}$ | $10^{9}$ |
| 20 | $10^{5}$ | $10^{9}$ |
| 30 | $10^{4}$ | $10^{8}$ |
| 40 | $10^{3}$ | $10^{6}$ |
| 50 | neg. | $10^{6}$ |
| 60 | neg. | $10^{6}$ |
| 70 | neg. | $10^{5}$ |
| 80 | · | $10^{5}$ |
| 90 | · | $10^{5}$ |
| 100 | · | $10^{4}$ |
| 110 | · | $10^{5}$ |
| 120 | · | $10^{5}$ |
| 130 | · | $10^{1}$ |
| 140 | · | $10^{1}$ |
| 150 | · | neg. |

· =not counted.
neg. =no growth in any dilution.

of fixing smears with methyl alcohol and staining by immersion for half to one minute in stevenel blue. The advantage of this over the old negative methods (i.e. the demonstration that turbid cultures show no bacteria stainable by Gram, etc.) is that it positively demonstrates the pleuro-pneumonia organism as a minute purplish spot or rod against

a blue background, often even in contaminated cultures; its advantage over the time-consuming Giemsa method is obvious when, perhaps, 100 smears have to be examined.

The writer is well aware of the inaccuracies inherent in dilution counts. The use of only one tube for each dilution can be severely criticised on purely statistical grounds. Indeed no suggestion is made here that anything other than gross differences can be demonstrated by this method; clearly a difference in end-point of one 1 in 10 dilution could be due either to a slight technical error or to a drop of 90 per cent. in the number of viable organisms. Nevertheless, the recorded results are reasonably consistent. In view of the complex life cycle of this organism with its conidioids, spheroids, filaments etc. it is indeed surprising that dilution counts are consistent at all; one might well expect that the breaking up of a cluster of, say, conidioids would throw dilution counts right out, but in fact this does not seem to happen; duplicate, triplicate or quadruplicate counts of the same culture by the same worker almost invariably give end-points within one 1 in 10 dilution of each other and frequently the end-points are identical.

It will be seen from Table I that, after seeding with young cultures, the maximum number of viable organisms is reached after 48 hours incubation and that thereafter there is a more or less steady decline. In this instance the organisms had died out after about 50 days at 37° C. and after about 150 days in the ice-chest. Actually, different strains vary somewhat in their viability; on occasion they may be found to be still viable after 70 days in the incubator but the majority of strains examined here behave approximately as shown in Table I. These results gave some standard of reference for comparison with the survival of dried organisms. It is worth noting here that Tang, Wei, McWhirter and Edgar (1935) found cultures viable for 45 days at 37° C. and for 98 days at 0° to 5° C.

## THE TECHNIQUE OF DRYING

Initial tests with the freeze-dryer showed that rapid drying of organisms suspended in horse serum resulted in a satisfactory product (i.e. the organisms remained viable for at least 200 days) provided it was kept in the ice-chest, but that the organisms failed to survive for more than a few days at 37° C. It was considered that, to be of practical value, it would be necessary to have a product which would survive for at least 12 months in the ice-chest followed by at least 2 months at a temperature of 37° C.

The problem was more difficult than was anticipated. A representative of Edwards & Co. considered it unlikely that complete drying could occur if the drying tubes were plugged with cotton wool since the wool acts as an inhibitor to the removal of water. Without cotton wool plugs it was impossible to maintain sterility and while complete sterility may not be essential with organisms

that can be re-isolated on solid media it is absolutely essential for the re-isolation of the pleuro-pneumonia organism which can only be satisfactorily regrown in liquid media. Furthermore it was pointed out that the removal of the final traces of water took a considerable time. The fact that the actual drying was incomplete in the initial experiments with the freeze-dryer explained why the original crude experiments were so surprisingly successful and finally led to the technique now adopted. In the original experiments small tubes containing the material to be dried were placed over phosphorus pentoxide in larger tubes; the large tubes were inefficiently evacuated by a few turns of the Gerrick pump before sealing; it is certain that such a procedure did not immediately remove all water and it is reasonable to suppose that the final drying was brought about by the phosphorus pentoxide in the large tube.

Using a centrifugal freeze-dryer the technique finally adopted is as follows: —

A given volume of the culture to be dried (usually 10 c.c.) is placed in a very tightly plugged, sterile centrifuge tube and centrifuged at 2500 r.p.m. for two hours. Taking sterile precautions the supernatant is discarded and the deposit, re-suspended in one-tenth (1 c.c.) of the original volume of sterile horse serum, is distributed either in bulk or in 0·1 c.c. amounts to sterile, plugged drying tubes. The tubes are placed in the centrifuge carrier plate assembly over three layers of phosphorus pentoxide and both centrifuge and evacuator are started. The centifruge is left running for two or three minutes only by which time the material is either frozen solid or is sufficiently turgid not to bubble. Evacuation is continued for 1½ to 3 hours according to the bulk of the material to be dried. At this stage the suspension *appears* to be quite dry but remains adherent to the side of the tube. It remains viable for months, and probably years, if stored in the ice-chest at this stage, but dies in a few days at 37°C. The tubes are now removed to a desiccator (which here was a McIntosh and Fildes anaerobic jar) over phosphorus pentoxide and the whole evacuated for about twenty minutes by attachment to one of the nozzles of the secondary drying header, the remaining 29 nozzles being sealed off with empty tubes. The sealed and evacuated desiccator is then placed in the ice-chest for about ten days, after which it is found that the dried material is largely free from the glass and can be readily shaken about. The tubes are then removed from the desiccator and constrictions drawn in the necks and a final evacuation is done for 30 minutes on the secondary drying header before sealing. This technique is simple and, as far as possible, ensures sterility from start to finish.

When a culture is required the dried material is reconstituted in 10 c.c. of serum broth and consecutive 1 in 10 dilutions are made for 6 to 12 tubes, depending on whether mere re-isolation or a count is desired. Usually a pure growth is obtained in the first tube but occasionally contamination occurs and the dilution technique has the effect of diluting out contaminants enabling one to obtain a pure culture somewhere in the series.

The results of a few tests using the above technique are shown in Table II. It will be seen that the cultures have survived storage for 475 days both in the ice-chest and at 37° C., from which it is evident that the technique is satisfactory. Other dried cultures are, in fact, still viable after storage in the ice-chest for over three years and it is anticipated that they will still be viable after ten years.

TABLE II.

THE RESULTS OF VIABILITY TESTS ON DRIED PLEURO-PNEUMONIA ORGANISMS STORED AT 37° C AND IN THE ICE-CHEST

| *Storage* | *Suspension diluted* 1 *in*— | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $10^1$ | $10^2$ | $10^3$ | $10^4$ | $10^5$ | $10^6$ | $10^7$ | $10^8$ |
| 200 days at 37° C ... ... | $+_3$ | $+_3$ | $+_4$ | $+_5$ | $+_5$ | · | · | · |
| 200 days at 5° C ... ... | $+_3$ | $+_3$ | $+_4$ | $+_4$ | $+_5$ | $+_5$ | $+_7$ | — |
| 245 days at 5° C ... ... | $+_2$ | $+_3$ | $+_4$ | $+_4$ | $+_6$ | $+_6$ | $+_7$ | — |
| 355 days at 37° C ... ... | $+_4$ | $+_4$ | $+_5$ | $+_{10}$ | — | — | — | — |
| 365 days at 5° C followed by 61 days at 37° C ... ... | $+_4$ | $+_5$ | $+_6$ | $+_7$ | $+_7$ | · | · | · |
| 365 days at 5° C followed by 110 days at 37° C ... ... | $+_3$ | $+_3$ | $+_4$ | $+_5$ | $+_5$ | $+_8$ | $+_8$ | — |
| 475 days at 37° C ... ... | $+_3$ | $+_4$ | $+_4$ | — | — | — | — | — |
| 475 days at 5° C ... ... | $+_2$ | $+_3$ | $+_4$ | $+_4$ | $+_7$ | — | — | — |

Figs. 4, 5, etc. =days after sowing that growth occurred.

The preliminary experiments on technique, including those referred to in Table II, were done with young organisms, i.e. collected after 48 hours incubation. This is the usual practice when drying any organism. It became desirable to know, however, if older cultures could be dried and maintained in a viable state. Accordingly cultures which had been incubated for seven days were dried and tested for viability from time to time. The results, shown in Table III, indicate that 7-day cultures survive for at least 150 days when stored in the ice-chest and that they die out in less than 101 days when stored at 37° C.

## THE EFFECT OF DRYING ATTENUATED CULTURES

The technique of testing dried cultures for virulence was as follows. The tube of dried material was opened and sown to serum broth as outlined above. As soon as a confirmed and pure growth was obtained a subculture was made to another tube of serum broth. This was incubated for the desired time, usually two days, and then injected into cattle intramuscularly in a dose of 1 c.c.

TABLE III.

THE SURVIVAL OF A DRIED 7-DAY CULTURE

| *Storage* | *Suspension diluted* 1 *in*— | | | | | |
|---|---|---|---|---|---|---|
| | $10^1$ | $10^2$ | $10^3$ | $10^4$ | $10^5$ | $10^6$ |
| 53 days at 37° C ... ... | $+_5$ | $+_5$ | — | — | — | — |
| 53 days at 5° C ... ... | $+_3$ | $+_4$ | $+_4$ | $+_5$ | — | — |
| 101 days at 37° C ... ... | — | — | — | — | — | — |
| 101 days at 5° C ... ... | $+_3$ | $+_4$ | $+_6$ | $+_8$ | — | — |
| 150 days at 37° C ... ... | — | — | — | — | — | — |
| 150 days at 5° C ... ... | $+_3$ | $+_3$ | $+_4$ | $+_5$ | $+_5$ | |

The first strain to be dried and subsequently used for vaccine seed material had been tested for virulence before drying by the injection of 1 c.c. of a 7-day culture into three cattle and found safe after the 43rd weekly passage through serum broth. After three more passages, the last of which was for two days only, the strain was dried and labelled F46. The dried material was stored in the ice-chest. When required for vaccine a tube was opened and sown to serum broth; when growth appeared subcultures were made and these were incubated for two days only before being used for seed material. Further subcultures were made each week, being incubated for two days and then stored in the ice-chest for five days. After 10 subcultures that particular line was discarded and a fresh tube of dried material opened. The vaccine (50 c.c. volumes) as then prepared was sown and incubated for two days, corked and sealed with paraffin and re-incubated for a further two days as a test of purity. Vaccine was, thus, a 4-day growth. It was stored in the ice-chest until issued or discarded. All vaccine not issued one month after storage in the ice-chest was discarded as time-expired. Under these conditions 198,025 doses were prepared and issued from December 1949 to June 1950. Only very few untoward results were reported. Nevertheless it was shown later that cultures made from the dried F46 material did, in fact, possess some virulence. Thus of three animals inoculated with a 7-day growth of the 2nd generation from the dried one developed a typical swelling and died 32 days later.

In December 1949 organisms from a 2-day growth from a second generation culture of F46 were collected and dried; tubes of these were labelled F48. Over 50 tubes were prepared and it was anticipated that these would supply seed material for vaccine production for at least 10 years.

In June 1950 the first tube of dried F48 was opened. From then until production ceased in October 1950 some 114,000 doses

of vaccine were prepared and issued. By October alarming reports were coming in of thousands of swellings and hundreds of deaths and it was clear that these could be traced to the inoculation of vaccine prepared from strain F48. As a result of these reports experiments on the virulence of dried organisms were carried out with the results shown in Table IV.

Table IV.

The Effect of Drying on the Virulence of Attenuated Cultures

| *Before drying* | | | *After drying* | | |
|---|---|---|---|---|---|
| *Strain* | *Generation number* | *Results in cattle* | *Strain* | *Number days dried* | *Results in cattle* |
| F | 43 | 0/3 | F46 | 1076 | 1/3 |
| | | | F48 | 219 | 3/4 |
| F48 | 14 | 0/3 | F62 | 101 | 1/2 |
| 139 | 45 | 0/3 | 139/51 | 18 | 2/3 |
| | 49 | 0/3 | | | |

Numerator =number cattle dying and/or showing swellings.
Denominator =number cattle inoculated.

Reference has already been made to the virulence of the F46 cultures.

After storage for 219 days in the ice-chest culture from the dried F48 was inoculated into four cattle; one animal, probably naturally immune, showed no reaction while the other three developed typical swellings and died 31, 36 and 44 days respectively later.

Weekly subcultures of the F48 strain were made for 14 weeks after which the organisms were dried and labelled F62. After storage for 102 days in the ice-chest a 7-day culture was inoculated into two cattle; one animal developed a slight swelling but rapidly recovered. The same strain tested before drying caused no reaction in any of the three inoculated animals.

Strain 139, tested after the 45th and again after the 49th subculture in serum broth and found safe on both occasions, was dried off as a 7-day growth at the 51st subculture and labelled 139/51. Culture tested for virulence after storage for 18 days in the ice-chest caused swellings in two of the three inoculated cattle and one of these died 32 days later.

From these results it seems evident that the drying of attenuated cultures even when 7 days old results in a sharp increase in virulence—a disappointing finding in that it meant that without some further modification standardisation of the seed material for vaccine production could not be achieved by drying.

## EXPERIMENTS WITH DRIED VACCINE

The dried vaccine used in these experiments was prepared as follows. A tube of F46, not then suspected of being virulent, was opened and subcultured five times, thus reaching the 51st generation. 50 c.c. volumes of serum broth were seeded and incubated for two days. The organisms were collected and dried. After 138 days in the ice-chest the dried material was re-suspended in plain broth to its original volume; actually the dried organisms from 40 c.c. serum broth were re-suspended in 40 c.c. plain broth. Within one hour of suspension cattle were injected intramuscularly with amounts of the vaccine varying from 5 c.c. to 1 c.c. As controls further cattle were inoculated with 1 c.c. normal serum broth vaccine prepared from a 3rd generation culture from the dried F48 also in the 51st generation. Two months later the surviving animals, together with two non-vaccinated controls, were tested for immunity against the standard culture 1178. Sometime before the test injection all animals were bled and tested for antibody by the complement fixation test (Campbell, 1938) and flocculation test (Priestley, 1951). The results of the experiment are shown in Table V.

Four of the 12 animals vaccinated with dried vaccine developed big swellings and two died, both after 42 days; sera from these animals together with that from one more (251) showed the presence of antibody. All the four animals inoculated with normal vaccine developed swellings and antibody and one of these died after 33 days. When the 10 survivors of the dried vaccine were tested for immunity only four of them survived; two of these (255 and 260) having developed swellings and one (251) having shown a response in that it produced antibody, might reasonably have been expected to be immune; the fourth survivor (261) may perhaps have been naturally resistant. The three survivors of the normal vaccine were, as was to be expected, solidly immune. The two controls died after 28 and 30 days respectively.

From these results it appeared evident that basically the vaccine was too virulent. It also seems evident that dried organisms, even comparatively virulent ones, have little chance of surviving in the animal body (at least when suspended in broth) and hence establishing an immunity; thus, 6 of the 12 inoculated animals showed no reaction to the vaccine, no antibody production and no immunity. Is seems probable, therefore, that had a safe dried vaccine been used the immunising effect would have been even less in evidence.

A second experiment was later carried out using the same dried material after storage in the ice-chest for 355 days. Unfortunately, some other infection killed off some of the experimental animals but of the four survivors none reacted to the vaccine and when tested for immunity three developed swellings and two died. It was clear that no immunity had been set up.

TABLE V.

THE RESULTS OF ATTEMPTED IMMUNISATION WITH DRIED VACCINE

| | | | Results | | | | |
|---|---|---|---|---|---|---|---|
| | | | After vaccination | | | After test | |
| Vaccine | Dose | Bull. no. | Antibody | Reaction | Days to death | Reaction | Days to death |
| dried | 5 c.c. | 249 | + | + | 42 | · | · |
| | | 250 | — | — | S | + | 23 |
| | | 251 | + | — | S | — | S |
| | | 252 | — | — | S | + | 29 |
| | 2 c.c. | 253 | + | + | 42 | · | · |
| | | 255 | + | + | S | — | S |
| | | 256 | — | — | S | + | 32 |
| | | 258 | — | — | S | + | 34 |
| | 1 c.c. | 254 | — | — | S | + | 27 |
| | | 259 | — | — | S | + | 27 |
| | | 260 | + | + | S | — | S |
| | | 261 | — | — | S | — | S |
| normal | 1 c.c. | 257 | + | + | 33 | · | · |
| | | 262 | + | + | S | — | S |
| | | 263 | + | + | S | — | S |
| | | 264 | + | + | S | — | S |
| controls | | 291 | · | · | · | + | 28 |
| | | 295 | · | · | · | + | 30 |

S = survived.

The results of these two experiments, while interesting, were disappointing from a practical point of view since it seems unlikely that the simple preparation of a dried vaccine will become a practical proposition.

## THE VALUE OF DRIED CULTURES

In spite of the fact that neither of the original objectives of the work have been achieved, drying is not without its uses.

Since drying raises the virulence of attenuated cultures it might be expected that dried virulent cultures would retain their viru-

lence. Experiment has shown this to be the case. Thus, for instance, strain 1178 dried as a 4th generation culture was tested for virulence in the usual way, i.e. inoculation of the 2nd generation culture from the dried material, after storage in the ice-chest for various lengths of time. The results are shown in Table VI. It is perhaps surprising to find such a high proportion of non-reactors (5 out of 15) but it would seem that, on the whole, the results are consistent enough. The mean time to death of the 10 animals which died was 26·7 ±1·4 days.

Other dried virulent strains have given similar results. Thus the use of a standard dried virulent culture at least permits comparable tests of immunisation procedures to be carried out at different times.

TABLE VI.

TIMES TO DEATH OF CATTLE INOCULATED WITH 1 C.C. CULTURE FROM THE DRIED STRAIN 1178

| *Days dried culture stored* | *Bull. no.* | *Days to death* |
|---|---|---|
| 15 | 271 | 30 |
| | 281 | 30 |
| 45 | 291 | 28 |
| | 295 | 30 |
| 93 | 315 | 33 |
| | 316 | 25 |
| 151 | 349 | S |
| | 351 | 27 |
| 186 | 390 | S |
| | 391 | 23 |
| 198 | 399 | S |
| | 400 | S |
| 217 | 417 | 20 |
| | 423 | 21 |
| 245 | 451 | S |

In view of these results and of those recorded in the last section on the use of dried vaccine the explanation of Daubney's (1938) reported failure to maintain the virulence of dried lymph becomes clear; it was undoubtedly due to his method of test, i.e. direct inoculation instead of inoculation following culture.

Dried cultures can also be used as the starting point for obtaining attenuated cultures for vaccine seed and provided the dried material is all from the same source it will be found that the safety point is reached after a more or less constant number of subcultures which is, quite definitely, not the case when lymph is used as the starting point. The saving in cattle is obvious. As an example, at different times tubes of F48 have been opened and tested for virulence after subculture; on two occasions the organisms were attenuated after 14 subcultures and on a third occasion after 18 subcultures.

## SUMMARY

Viability tests on serum broth cultures of the contagious bovine pleuro-pneumonia organism indicate that generally it survives for about 50 days when stored at 37° C. and for about 150 days when stored in the ice-chest.

A technique for drying the organism *in vacuo* over phosphorus pentoxide is described in detail. It has been shown that dried organisms remain viable for at least three years in the ice-chest and for at least 475 days in the incubator at 37° C.

Evidence is brought to show that the drying of attenuated cultures results in a sharp increase of virulence.

Conversely, as might be expected, dried virulent strains retain their virulence.

Experiments with dried vaccine have led to the conclusion that this is not yet a practical proposition.

## ACKNOWLEDGMENTS

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# INVESTIGATIONS INTO DELAYED TOXICITY DUE TO DIMIDIUM BROMIDE

## I. Field Observations

By

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### INTRODUCTION

During March and April, 1950, some 12,600 head of cattle were inoculated with dimidium bromide in the European settled areas of the Sotik highlands, Kenya. These lie 35° E., less than 1° S. of the equator. The altitude varies between 6,000 to 7,000 feet and the average yearly rainfall is 40 to 50 inches. The object of this mass inoculation was to combat trypanosomiasis, which was being spread by flies mostly in the absence of Tsetse and was estimated, by sampling, to have an incidence of about 10 per cent. in the cattle population.

All adult bovines were inoculated intramuscularly in the back of the thigh with 0·3 g. dimidium bromide dissolved in 10 ml. water. An animal weighing 300 Kg. (estimated as an average weight) thus received 1 mg./Kg., whereas animals weighing 350 to 400 Kg., such as working oxen and older females, received only 0·8 to 0·75 mg./Kg.

Bell (1945) reported 0·8 mg./Kg. as the minimum curative dose and Wilde (1949) found 0·75 mg./Kg. to be effective in the treatment of *T. congolense* infection. Randall and Laws (1947) in Uganda stated that a single dose of 1·0 to 1·5 mg./Kg. was effective and unlikely to cause photosensitisation or local lesions. Stewart (1947) in the Gold Coast stated that for field use a dose of 1 mg./Kg. was employed. Wilson (1948), however, noted three relapses in a group of eighteen animals dosed at 1 mg./Kg.

As 3,000 doses of the drug had been successfully used in the district during the previous three years, no untoward sequelae were expected. Nevertheless, it is estimated that 6 per cent. of animals treated at Sotik in 1950 developed a severe local reaction which consisted of marked local swelling and, later, sloughing of the skin. Six weeks after commencement of the inoculation campaign, symptoms of photophobia together with necrotic dermatitis were manifest in a number of animals. The condition was recognised as photosensitisation by the District Veterinary Officer.

### ANIMALS AFFECTED

In general, the animals most affected were light coloured Zebu and low-grade stock, in contra-distinction to high-grade and exotic breeds which did not suffer severely. Exceptions to this were noted in that some completely black animals did go down with

the condition, but these were always mild cases. Again, a high-grade Friesian herd had many cases, probably due to the fact that the cattle were just recovering from an outbreak of foot and mouth disease. Working oxen and older cows and those in advanced pregnancy were most commonly and severely affected. According to information supplied by the Livestock Officer of the district, the number of reactions was as follows: —

| *Total Inoculated* | *Mild Reactions* | *Severe Reactions* | *Deaths* |
|---|---|---|---|
| 12,606 | 992 | 501 | 185 |
| | 7·8 per cent. | 3·9 per cent. | 1·4 per cent. |

The number of deaths and severe reactions would probably have been substantially reduced had farmers, on the appearance of symptoms, taken steps to assist the animals to combat photosensitisation.

### SYMPTOMS

Symptoms usually appeared between the fortieth and fiftieth days after inoculation. They were never observed before this period, but two cases of severe photosensitisation occurred after a delay of seventy days post-inoculation. Animals reacting mildly showed signs of sensitivity and irritation of the muzzle and of the skin of the face and ears, together with slight lacrymation.

The symptoms in severe reactors were loss of condition, photophobia and lacrymation. There was intense irritation and hypersensitivity of the skin—usually of non-pigmented parts exposed to direct sunlight—accompanied by oedematous swellings of the muzzle, face, backs of the ears, base of the horns and dewlap, followed by serous exudation with hardening and cracking of the skin over these areas. Similar changes were seen along the back, flanks, medial and lateral aspect of the thigh, escutcheon and the soft skin of the udder and teats in cows. No lesions were seen below the carpus and tarsus. Strips of "boarded" skin peeled off the back, flanks and udder exposing a raw granulating surface. The teats and udders of some cows were so severely affected that the former became necrotic and dropped off. The skin on the ears also peeled leaving them raw and mis-shapen. With the hardening of the skin over the back and flanks the gait was affected and the animals walked stiffly. The general demeanour was depressed, the animals did not feed and were often constipated. In severe cases clinical icterus developed early and deaths occurred 7 to 14 days after the onset of symptoms. Many in-calf cows aborted. On three farms at least, calves born of dams which showed various degrees of photosensitisation during the period of reactions, also developed lesions of the muzzle, ears and back during the first week of life. No deaths were recorded among calves and no similar condition had been seen previously on these farms.

### POST-MORTEM FINDINGS

The following general picture was presented at post-mortem examination by animals dying from delayed toxicity. The skin lesions were similar to those described above. In some cases deaths occurred before large areas of skin had peeled away. There was emaciation and marked general icterus. The liver was enlarged and friable, with yellow discolouration. The kidneys were slightly enlarged and dark-brown to black in colour. The lungs were hyperaemic and oedematous. The bowel usually contained hard, dry faeces. No material from these cases was available for study.

### PASTURE EXAMINATION

As it was considered possible that the photosensitisation may have been partly due to the ingestion of plants containing toxic substances, an examination of the grazing areas in the district was undertaken. The following species belonging to families known to be harmful were collected: *Lippia asperifolia, Lantana salvifolia, Hypericum* spp. *Trifolium usambarensis, T. semipilosum.*

*Lippia rehmanni* and *L. pretoriensis* have also been shown by Quin (1933) to possess an icterogenin which also produced photosensitisation. *Lantana camara,* according to Steyn and Van der Walt (1941), causes photosensitisation and icterus. *Lantana salvifolia* was stated by Walker (1928) to be non-toxic for sheep. Mettam (1933), however, claimed to have killed sheep by feeding this shrub. Various species of *Hypericum* are known to cause photosensitisation without clinical icterus. Certain *Trifolium* spp. if ingested in large quantities produce photosensitisation (Hausemann and Haxthausen, 1929).

At the time of the investigations reported herein there was ample grazing which consisted mainly of *Themeda triandra, Eragostis* spp., *Andropogon* spp., and *Setaria* spp.; it is unlikely, therefore, that stock would have grazed the *Lippia* and *Lantana* bushes and no evidence of such grazing was in fact found. The *Hypericum* spp. was a small creeping herb found in dense bush undergrowth where it was virtually impossible for stock to graze. Two *Trifolium* spp. occurred in large numbers in the lower lying wet areas but cases of photosensitisation were not confined to cattle grazing there.

### DISCUSSION

The factors which predispose cattle to delayed reactions following the administration of dimidium bromide are, as yet, largely hypothetical. Stewart (1947), in the Gold Coast, and Evans (1948), in the Sudan, both found that the poor grazing associated with prolonged drought seemed to be of some importance. The cattle described by Wilde (1949) were also stated to have been grazed on recently cleared bush with many weeds. The pastures in the Sotik area were green and apparently of high nutritive value both prior to and during the course of this outbreak of photosensitisa-

tion. No indication that toxic plants were of significance was obtained from a careful examination of pastures in the area.

Evans (1948) remarked that cattle in good condition showed the most severe reactions, but in the outbreak here recorded, it was observed that in a high-grade Friesian herd, the loss of condition, caused by an attack of foot-and-mouth disease, was associated with unusually severe toxic symptoms. Wilde (1949) noted that delayed reactions tended to be particularly severe in animals in an advanced stage of pregnancy and in those which had calved recently.

The skin lesions observed in high-grade calves born of dams which were showing symptoms suggest either a transfer of the circulating sensitising agent via the placenta and/or the colostrum, or an intra-uterine liver injury in the calf. We have no doubt as to the nature of the lesions which were observed independently on at least three farms under close European supervision, and seen by us personally.

The relatively high susceptibility of native-type (Zebu) as opposed to exotic breeds of cattle has not been seen so clearly previously, as most reported inoculations have been in African-farmed areas, or in experimental groups of Zebu-type animals. The difference was invariably noted in this outbreak on farms where Zebu work-oxen and grade milking cattle were inoculated simultaneously. The strength of solution and route of inoculation have not been shown to be of significance in determining whether reactions will or will not occur. The minimum dosage recorded by various authors as followed by delayed toxicity within a constant period of about six to seven weeks is as follows: 1 mg./Kg. (Evans, 1948), 1·5 mg./Kg. (Wilson, 1948; Wilde, 1949), 2 mg./Kg. (Randall and Beveridge, 1946, 1947; Stewart, 1947), 3 mg./Kg. (Bell, 1945). In this outbreak, reactions were recorded in some 11·7 per cent. of over 12,000 animals inoculated with approximately 1 mg./Kg. of the drug intramascularly, but in a large proportion of the bigger adult animals dosage was, in actual fact, nearer 0·8 mg./Kg.

In subsequent papers an account will be given of work designed to show the influence of dosage and method of administration of the drug on delayed toxicity. The effect of various pastures also requires investigation, together with the relationship of the consistent liver lesion and frequent jaundice, to the development of photosensitisation.

SUMMARY

Delayed reactions characterised by skin lesions typical of photosensitisation are reported in 6 per cent of cattle inoculated during 1950 with dimidium bromide.

An examination of the pastures failed to reveal any poisonous plants which might have produced photosensitisation.

Other factors which might have played a part are discussed.

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# INVESTIGATIONS INTO DELAYED TOXICITY DUE TO DIMIDIUM BROMIDE

## II. The Effects of the Re-administration of Dimidium Bromide to Zebu Cattle

By

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### INTRODUCTION

An outbreak of photosensitisation following mass inoculation of cattle with dimidium bromide has been described in the first communication of this series (Thorold and Plowright, 1952). The results of the re-inoculation of a group of the animals are reported in the present paper.

### METHODS

*Experimental animals.* Twenty-six Zebu-type native cattle were purchased from European settlers in the Sotik area, Kenya, and put on to partly cleared natural grazing situated on a local farm. Six had shown symptoms of photosensitisation after previous inoculation with 1 mg./Kg. of dimidium bromide (S. 21 to 26 inclusive); the others had received dimidium bromide but had remained well. Of the six, at least one (S.22) had been seriously affected, as it still had large scarred areas on both flanks where the skin had not yet completely grown. Half of the cattle were females (cows); the rest were castrated males. They were divided into three groups as follows: —

Group I —three females, seven males.
Group II —four females, six males.
Group III—six females.

Group III comprised those which had previously shown delayed toxic symptoms. Because the great majority of field cases were in female animals this group contained no males. Animals of all colours were present (Table I).

*Experimental treatment.* On 14th July 1950 (day 1), all the cattle were given dimidium bromide, using a separate needle for each animal. Groups I and II received the drug intramuscularly in 3 per cent. solution in distilled water at the rate of 1 mg. and 2 mg./Kg. respectively; Group III was divided into two subgroups, half of which received 1 mg./Kg., the other half 2 mg./Kg.

Twenty days after inoculation all the cattle were in fair condition with the exception of one or two in Group III which had not fully recovered from the first reaction and were still poor. After this examination, half of each group was transferred by rail to Kabete where they were housed for ten days. They were then turned out into small, intensively-managed paddocks in which no

shelter was provided. The grazing was predominantly green, mature Kikuyu grass (*Pennisetum clandestinum*). Near the time of the onset of reactions, the grasses had begun to dry although the sun was seldom seen for at least a fortnight preceding the reactions, being obscured by heavy, low cloud. The rainfall during the period of pasturage at Kabete was negligible and a bright sunny period succeeded early in September, after which no further cases arose. During the time preceding photosensitisation dew was not heavy and the days were not cold. Meanwhile, at Sotik the weather during the period was characterised by cool, dull mornings up to about 08.30 hours, hot sun following until about 15.30 hours. The evenings were cloudy and the nights were generally cold, with heavy dew.

*Technique.* Frequent clinical examinations of the Kabete animals were made in the stall, at pasture and in the crush at the times of bleeding. Blood was taken from the jugular vein on eight occasions from day 27 to day 91 inclusive (Table II). The following biochemical determinations were carried out:

(*a*) Van den Bergh reaction by the method described in Kolmer and Boerner (1945);

(*b*) Haemoglobin as acid haematin, comparing the colour developed after forty minutes to that of a Newcomer Disc in a Klett colorimeter (Newcomer, 1923). Sodium oxalate 2 mg. per ml. was dried into tubes as the anticoagulant.

(*c*) Total Serum Protein—by the specific gravity method of Phillips (1945) using sixteen standard copper sulphate solutions of specific gravity 1,018-1,032.

## RESULTS

### *Clinical Observations*

Table I shows the frequency and degree of photosensitisation observed, together with the time of its first detection.

To summarise, of twenty-six animals re-inoculated, nineteen (73 per cent.) showed symptoms suggestive of photosensitisation. Of these, eleven were maintained throughout the experimental period at Sotik while the remainder were transferred to Kabete twenty days after the inoculation with dimidium bromide. Severe toxic symptoms occurred in six none of which had showed signs of photosensitisation with the first treatment, but in this experiment all had received dimidium bromide at the higher level of 2 mg./Kg. Four were stationed throughout at Sotik, two latterly at Kabete. One animal died (No. S.16); another (No. S.2) would probably have done so but was destroyed. The mortality in this trial was, therefore, approximately 7·7 per cent., allowance being made for the fact that no measures were taken to protect the cattle from direct solar action.

TABLE I

| *Animal No.* | *Colour* | *Sex* | *Dosage* | *Degree of Photosensitisation* | *Result ; time of occurrence (in days after inoc.)* |
|---|---|---|---|---|---|
| *Group Ia*—Moved to Kabete. | | | | | |
| S.1/372 | Light fawn with white mottling | M | 1 mg./Kilo | Nil | — |
| S.3/376 | Black and tan ... ... | M | ,, | Nil | — |
| S.4/373 | Black ... ... ... | M | ,, | Doubtful | 51 days |
| S.5/378 | Black, grey and white patches | F | ,, | Nil | — |
| S.8/366 | Red brown Ankole ... | F | ,, | V. mild | 51 days |
| *Group Ib*—Retained throughout at Sotik. | | | | | |
| S.12 | White ... ... ... | F | 1 mg./Kilo | Very mild | 41 days |
| S.14 | Black ... ... ... | M | ,, | Very mild | 41 days |
| S.17 | Red ... ... ... ... | M | ,, | Very mild | 45 days |
| S.18 | Black and white ... ... | M | ,, | Nil | — |
| S.20 | Dark brown ... ... | M | ,, | Mild | 45 days |
| *Group IIa*—Moved to Kabate. | | | | | |
| S.2/369 | Red, brown, white ... ... | M | 2 mg./Kilo | Severe | Killed 48 (?) days |
| S.6/375 | Black ... ... ... | M | ,, | Mod. Severe | Killed 48 (?) days |
| S.7/374 | Black and tan ... ... | M | ,, | Nil | — |
| S.9/367 | Brown, white mottling ... | F | ,, | Mild | 48 (?) days |
| S.16/370 | White Ankole ... ... | F | ,, | Very mild | 52 days |
| *Group IIb*—Retained throughout at Sotik. | | | | | |
| S.11 | Black ... ... ... | F | 2 mg./Kilo | Very mild | 46 days |
| S.13 | Light tan ... ... ... | F | ,, | Mod. severe | Killed 40 days |
| S.15 | Red ... ... ... | M | ,, | Severe | 39 days |
| S.16 | Dun, white face ... ... | M | ,, | Severe (died) | 39 days |
| S.19 | Red ... ... ... | M | ,, | Mod. severe | 41 days (?) |
| *Group IIIa*—Moved to Kabete. | | | | | |
| S.22/368 | White/grey ... ... | F | 1 mg./Kilo | Nil | — |
| S.23/377 | Red/Brown ... ... | F | 2 mg./Kilo | Mild | 51 days |
| S.24/371 | Fawn ... ... ... | F | 2 mg./Kilo | Very mild | 51 days |
| *Group IIIb*—Retained throughout at Sotik. | | | | | |
| S.21 | Grey ... ... ... | F | 1 mg./Kilo | Mild | 45 days |
| S.26 | Sandy ... ... ... | F | 1 mg./Kilo | Moderate | 41 days |
| S.25 | Sandy ... ... ... | F | 2 mg./Kilo | Nil | — |

The interrogation mark after the latent period in the case of bovines Nos. S.2, S.6, S.9, refers to the fact that these animals were first observed clinically affected on the fiftieth day. They had progressed, however, to a stage which indicated a duration of at least forty-eight hours. A similar mark is inserted after No. S.19 as definite symptoms of photosensitisation proper were not observed at this date, but were seen five days later. Earlier symptoms included tucked-up abdomen and staring coat.

*Symptoms*

No obvious changes were observed until the onset of delayed toxicity. At first all the animals transferred to Kabete appeared to improve somewhat in condition, especially those of Group III.

The first symptom commonly seen was a hyperaemia of the sub-epithelial tissues of the muzzle; this became drier than normal and exuded droplets of serum which coagulated into flakes. The change commenced near the nares and occurred even on black muzzles. At the same time there was often a slight watery discharge from the eyes. The conjunctiva was sometimes slightly injected and jaundiced in severe cases. Photophobia was not observed, nor was closure of the lids. The cornea remained unaffected. A mucous nasal discharge, which dried in the nares or on the muzzle, was observed, especially in severely affected animals, and dust collected on the sticky surface. Smacking of the lips and salivation were evident in S.2 and S.6, but no explanation of these symptoms could be found, even at post-mortem examination. Cunningham, Hopkirk and Filmer (1942) attributed the salivation noted in cattle with facial eczema to the licking of the nasal discharges, thus exposing the lingual mucosa to the rays of the sun.

Lesions were often observed on the ears, even on those which were black. These appendages were hyperaesthetic, the animal resenting handling and twitching them continuously. The skin on the edges and/or the base of the ear was thickened and hot. Eventually, these areas hardened and, where mobile, suffered a loss of elasticity resulting in cracking and the exudation of serum. The edges curled backwards characteristically. Hyperaemia, exudation and hypersensitivity at the base of the horn were also observed with consequential head shaking. Similar lesions developed in the body usually on unpigmented areas. Swelling and hardening of the skin of the dewlap was noted and was probably partly due to the gravitation of toxic inflammatory exudates from higher parts of the fore hand. No teat or udder lesions were observed in any of these animals although reported as occurring in field outbreaks.

In mild cases, even when not stabled, the lesions regressed rapidly and it is probable that many such cases passed unnoticed in the field, especially if examination was infrequent or cursory. After four to five days, peeling of exudate on the muzzle commenced and epithelial regeneration soon followed (5 to 7 days). The ear lesions, especially the turning back of the edges, persisted for much longer (3 to 4 weeks or more). Loss of condition was marked and rapid in severely affected animals and constipation was frequent.

An interesting observation made on these cattle further supports the diagnosis that the condition was a photosensitisation. The animals were branded on the haunch as soon as they arrived at Kabete and the brands cicatrised normally. However, in those

TABLE II

RESULTS OF VAN DEN BERGH TEST ON ANIMALS BROUGHT TO KABETE.

| *Number* | *Dose Kg.* | *Previous Photosensitisation* | *Day* 27 | 38 | 47 | 52 | 59 | 70 | 76 | 91 | *Extent of Photosensitisation* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| S4/373 | 1 mg. | none | — | — | — | + | — | + | + | — | Doubtful at day 51 |
| S8/366 | 1 mg. | ,, | — | — | + | + | + | — | — | — | Very mild at day 51 |
| S2/369 | 2 mg. | ,, | — | — | + | | (K day 48) | | | | Severe at day 48 |
| S6/375 | 2 mg. | ,, | — | — | +(5) | | (K day 49) | | | | Mod. severe at day 48 |
| S9/367 | 2 mg. | ,, | — | — | — | + | — | — | — | — | Mild at day 48 |
| S16/370 | 2 mg. | ,, | — | — | — | + | + | + | + | — | Very mild at day 52 |
| S23/377 | 2 mg. | Present | — | — | — | — | — | — | — | — | Mild at day 51 |
| S24/371 | 2 mg. | ,, | — | — | + | + | + | — | + | — | Very mild at day 51 |
| S1/372 | 1 mg. | None | — | — | — | — | — | — | — | — | None |
| S3/376 | 1 mg. | ,, | — | — | — | — | + | + | — | — | ,, |
| S5/378 | 1 mg. | ,, | — | — | — | — | — | + | + | — | ,, |
| S7/374 | 2 mg. | ,, | — | — | — | — | + | + | — | — | ,, |
| S22/368 | 1 mg. | Present | — | — | — | — | — | + | — | — | ,, |

Figures for days refer to period since second treatment with dimidium bromide.
+ = Positive direct.
+5 = Positive direct and indirect of 5 mg./100 ml. bilirubin.
K = Slaughtered.

animals which became photosensitive and to a degree dependent on the severity of the reaction, the brand scars were "reactivated". The scabs were sequestrated by exudation of viscous yellow drops of serum from beneath. A similar activation of old lesions of photosensitisation was noted in Group III, that is in animals reacting a second time.

### *Biochemical Findings*

At Sotik daily clinical examinations were made, but only one sample of blood was obtained, on day 25, from the animals and all were negative for Van den Bergh reaction, whilst the haemoglobin ranged from 8·7 to 13·9 g./100 ml., and serum proteins were between 7·0 and 8·1 g./100 ml.

The results of the Van den Bergh reaction carried out when the animals were at Kabete, correlated with the onset of symptoms of photosensitisation, are given in Table II.

### *Evaluation*

Mention must be made of the high carotene content of normal bovine sera which made the direct reaction a little less sensitive than in species with water-clear sera, so that some animals may have been positive earlier but were not detectable for this reason.

All except one of the clinical cases developed a positive Van den Bergh reaction and in the slaughtered animal, S.6/375, the high figure for indirect bilirubin of 3·2 mg./100 ml. was recorded.

Four out of five cases showing no symptoms were also V.d.B. positive for a maximum of eleven days, indicating mild liver damage. All this group were normal at day 91. The estimations of the haemoglobin content and of the total serum proteins which were carried out in parallel with the Van den Bergh tests revealed no significant changes, the figures for each falling within the normal range throughout.

### *Post-mortem Findings*

Four animals came to post-mortem examination, of which only one died naturally (S.16). All were serious cases with the exception of S.13—which was recovering.

| *Animal No.* | *Post mortem Examination* | *Days of Reaction* | *Icterus* | *Liver Damage* | *Skin Lesions* |
|---|---|---|---|---|---|
| S.6/375 | Day 50 | 2(?) | ++ | ++ | + |
| S.2/369 | Day 53 | 5 | + | + | +++ |
| S.16 | Day 50 | 11 | +++ | +++ | ++ |
| S.13 | Day 61 | 21 | — | + | + |

### *Macroscopic Examination*

Jaundice of varying intensity was present in mucous and serous membranes, subcutaneous tissues, synovial membranes, kidney, heart, lungs and lymph glands of the first three animals.

The liver was swollen, congested, and a fine, regular, yellowish or greenish mottling showed through the capsule, or on incision. The liver appeared to be uniformly affected, the lesion never being more marked in one lobe than another. In S.16 and S.6 the parenchyma was obviously highly degenerate, friable and soft. The gall bladders were moderately full, containing clear green bile.

The spleen was enlarged and firm, but the Malpighian bodies were not prominent. In S.2 the pulp was somewhat brownish. At Kabete no gross macroscopic abnormality was noted in the kidneys, with the exception of bile staining which was more prominent in the medulla. In S.16 (Sotik), the kidneys were reported to be blackish, but this may have been associated with post-mortem changes. The urine of S.16 was deep blackish-green and that of S.6 was bile-stained. Small sub-endocardial ecchymoses were present in S.6, possibly due to slaughter by shooting.

Nothing of note was found in the intestinal tract, but signs of constipation were observed in the first three animals. There was a generalised oedematous enlargement of most of the lymph glands of S.2, which, however, also had extensive skin lesions of some days' duration. Oedema of the lungs was found in S.16.

### *Histo-pathology*

*Liver.* This organ was invariably affected and showed primarily a fatty degeneration of periportal distribution. Accompanying the degeneration was a severe congestion, usually also limited to the portal tracts and the immediately adjacent parenchyma. The extent of the fatty changes varied widely and was not entirely commensurate with the severity of the clinical symptoms, e.g., it was of limited extent in S.2/369; in S.16 practically every liver cell contained fat globules, although the periphery showed by far the most advanced fatty change.

Internal to the zone of primary fatty degeneration the hepatic and von Küpfer cells revealed an increasing accumulation of a granular, golden-brown pigment which appeared first near the nucleus and gave no reaction for iron. The amount of pigment was greatly reduced in paraffin stained sections—sometimes it was almost completely removed by the alcohols. It was noted that only frozen sections consistently gave a true picture of the extent of the liver damage. In S.16 the accumulation of the pigment, probably bilirubin, was such that necrosis of the hepatic cells occurred in foci of varying size, which usually lay contiguous with the central vein, and was followed by infiltration of dense aggregations of polymorphonuclear leucocytes (Fig. 1). Iron-free pigment was also found in the von Küpfer cells towards the periphery of the lobule and in macrophages of the portal tracts. In S.16 there were areas of eosinophilic necrosis of irregular size and shape near the central veins; these did not contain any accumulations of bile pigment,

nor had polymorphonuclears appeared in them. In the other animals degeneration of a few scattered cells around the central veins was noted, but not as severe as in S.16. The central veins appeared frequently to be dilated, but not distended with blood; except in No. S.16 this may have been due, to some extent, to the method of slaughter and bleeding out.

There was evidence of bile duct proliferation, of an extent dependent on the degree of fatty degeneration. The portal tracts were widened and showed compact cords of cells apparently derived from the bile ducts and growing out into the affected parenchyma. There were also a few small accumulations of mononuclear cells similar to those described in many conditions where degenerations occur within the liver lobule. The fibroblasts and connective tissues were possibly slightly increased. No permanent increase in the size of the portal tracts was noted, however, because a severe field case which had recovered had no cirrhosis. The reticulin framework of the liver remained intact and, so long as sufficient undamaged liver cells remained to allow the animal to survive, complete regeneration could occur with no scarring or cirrhosis. In S.16 the portal tract showed haemorrhages and increased reticulin network (Fig. III). In one animal (S.13) definite signs of a regenerative process were seen and fairly numerous large hepatic cells with deeply staining cytoplasm and a hyperchromatic large nucleus were visible around the portal tracts.

*Kidney.* No consistent abnormality was found.

*Spleen.* In the three animals examined (S.2, S.6, S.13) there was a significant reduction in the number of lymphoid cells present. The red pulp was congested. The Malpighian bodies were not prominent and their germinal centres were never actively lymphopoietic; around them were often large numbers of polymorphonuclears. In all cases, especially S.13, there were large quantities of iron-containing pigment in fixed and free macrophages, particularly those around the Malpighian corpuscles.

*Lymph Glands.* These showed a diffuse lymphoid hyperplasia of the cortex, sometimes extending to the medullary cords in S.2, S.6 and S.13. Non-iron-containing pigment was frequently present in the free and fixed macrophages, particularly of the medullary cords.

No abnormalities were noticed in the following organs: myocardium, adrenal, bone marrow and brain.

*Skin.* The skin changes were studied in sections of the ear of one animal (S.2). The inner aspect, with numerous glands of a sebaceous type, was normal, but the tip and external surface had marked changes associated with photosensitisation. The epithelium was intact with no pigmentation of the germinal layer and a complete loss of the granular layer: hence there was no keratinisation. At the same time the basement membrane of the epithelium was indistinct due to the formation of dense fibrovascular granulation

tissue in the dermis. At some points haemorrhage had occurred from the numerous new capillary blood vessels of the corium.

Overlying the epithelium was a thick, easily detached layer of dried exudate consisting largely of fibrin and polymorphonuclear leucocytes; the nuclei of the latter contributed to form dense masses of granular debris. Superficially, there was an indistinct layer in which swollen epithelial cells could be distinguished and also many cells with numerous yellowish-brown pigment granules. Similar granules occurred in large stellate cells of the subepithelial tissues and all could be bleached by potassium permanganate. It was thus found that even pigmented skin could be affected, the essential process being a sloughing of the superficial layers of the epidermis, with a sparing of the *stratum germinativum*.

### *Biochemical Observations on Slaughtered Animals*

Two litres each of urine and serum from Nos. S.2/369 and S.6/375 were extracted by the usual methods to remove porphyrins. No fluorescence was detectable in 10 per cent. hydrochloric acid extracts by ultraviolet light; spectroscopic examination was not possible at the time.

A heavy proteinuria was also found in both these animals. The livers of S.2/369 and S.6/375 had 19·9 per cent. and 21·5 per cent. of fat respectively. These determinations were carried out by mincing, freeze-drying and grinding the liver, followed by extraction with ethyl ether in Soxhlets in duplicate.

## DISCUSSION

According to the classification of Clare (1950), the photosensitisation occurring after the administration of dimidium bromide to cattle would fall into his group due to "failure of an elimination or detoxication mechanism", in this instance, more specifically, hepatic dysfunction. Evidence for this is brought forward in the findings that all except one of the clinical cases developed a positive direct Van den Bergh reaction and that a consistent liver lesion of an unusual and characteristic type was found in all affected animals from which tissues were obtained. These changes were often accompanied by clinical icterus. That animals which received as little as 1 mg./Kg. of the drug suffered some degree of liver damage, without photosensitisation, was attested by the finding of a positive Van den Bergh reaction in four of five animals out of a group of thirteen which remained apparently normal. The maximum duration of the positive reaction in these four cases was eleven days. The absence of any significant fall in the haemoglobin values, together with the immediate, direct positive Van den Bergh reactions, pointed to a jaundice of the obstructive type.

Conclusive proof of the cause of the photosensitisation necessitates the recognition in the blood of a photodynamic substance. In

all similar known instances phylloerythin and, possibly to a lesser extent, coproporphyrins, have been suspected to be the photodynamic agent. Further work on this point is under consideration at Kabete.

The liver lesion consisted essentially of a fatty degeneration of the periportal zone, accompanied by a swelling of the hepatic cells which resulted in constriction of the sinusoids and biliary canaliculi. Pigment, therefore, accumulated centrally and secondary degenerative changes made their appearance in the liver cells. The histology in the present condition does not correspond closely with that of the other members of this group of photosensitising diseases. Thus Theiler (1918) described "irregular" degeneration and necrosis of liver cells in Geeldikkop, and noted that individual cells or small groups of cells appeared "transparent". Sometimes centrilobular stasis and atrophy was observed; proliferation of bile ducts and increase of connective tissue in the portal tracts was present to a degree which appeared excessive in relation to the degree of liver damage. Fat was rarely present and then in moderate amount. Cunningham, *et al.* (1942), in describing a facial eczema affecting sheep, also gave data on the condition in cattle. Their description corresponds very closely to the clinical picture of delayed dimidium bromide poisoning; in addition, they made the very interesting observation that apparently normal lambs sometimes showed at post-mortem a liver lesion identical with that seen in cases of facial eczema. Histologically, the liver in mild cases of facial eczema shows occasional necrotic cells. A few polymorphonuclears may also be present and these are more frequent in the sinusoids. There is an increase of the portal connective tissue and many new "sprouts" from the bile ducts. In more severe cases, necrosis of the wall and obliteration of the lumen of medium-sized bile ducts occurs and in advanced cases there is "pressure atrophy" due to fibrosis of the portal tracts. Larger bile ducts may show desquamation of the necrotic lining epithelium. These severe reactions are more often present in the left lobe which becomes shrunken and later the right lobe may, on recovery, show compensatory hypertrophy. The liver lesions found in facial eczema are accepted as almost pathognomonic.

*Lechuguilla agave* poisoning is, according to Matthews (1938), accompanied by degeneration and fatty infiltration of the liver, with necrosis and cast formation in the bile ducts. The plant also produces a toxic effect on the kidney, both the glomeruli and the tubules. Bell (1947) implied that the liver lesion observed by him in Uganda in photosensitised animals following dimidium bromide administration, was mainly centrilobular. He also mentioned the increase in the bile ducts. Wilson (1948) noticed the periportal location of the fatty zone and cellular infiltration of the portal tracts.

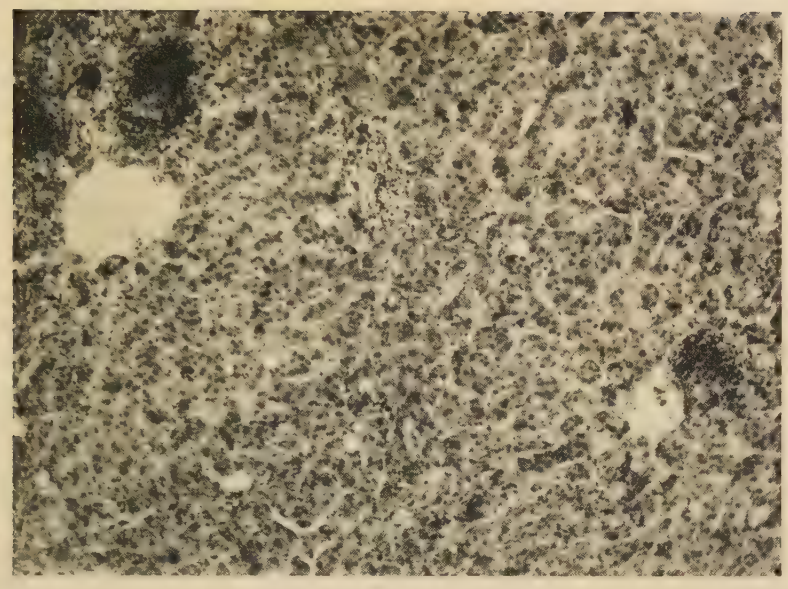

Fig. 1. Bovine S.16 : 2 mg./Kg. dimidium bromide intramuscularly. Died day 50 p.i. Typical case of photosensitisation. Liver—frozen sections, Sudan IV—haematoxylin.

Note two large dark foci of necrosis with accumulation of bile pigment and polymorphs. The fat infiltrated cells appear slightly darker and are in the periportal zones. (×100.)

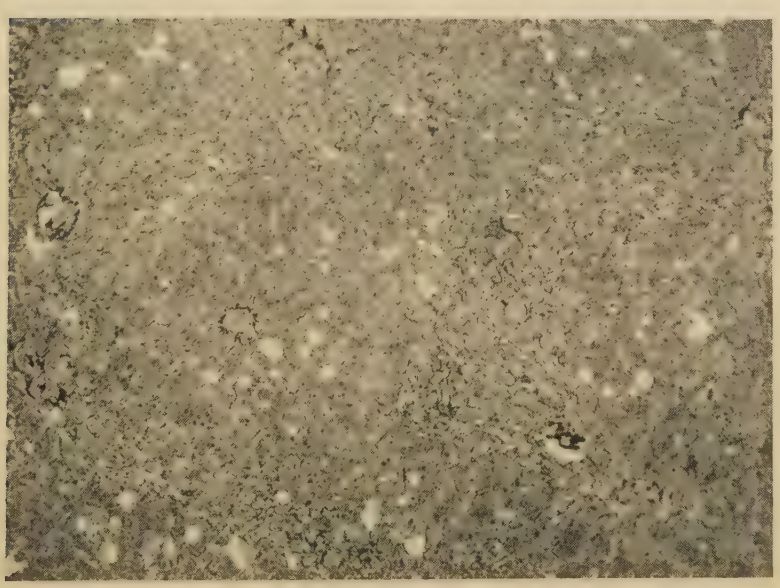

Fig. 2. Normal bovine liver—Wilder's reticulin stain. (×100.)

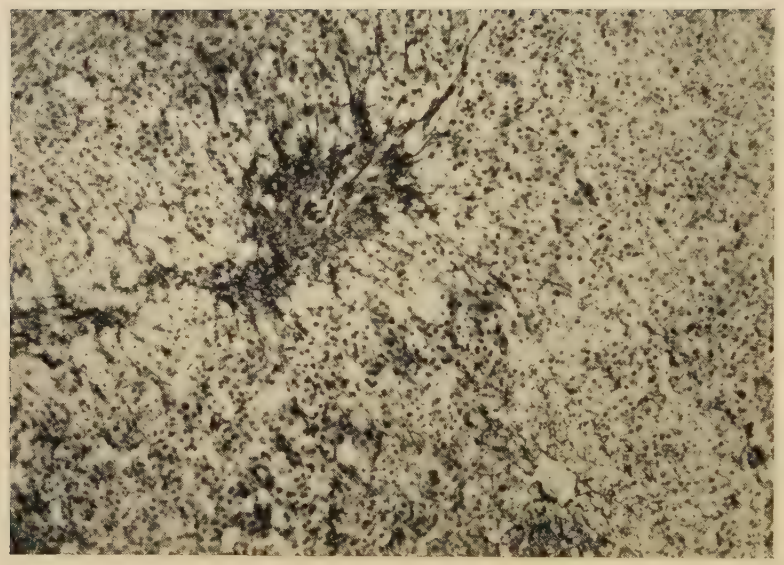

FIG. 3. Bovine S.16 : Liver—Wilder's reticulin stain. Portal tract cut obliquely showing haemorrhage and increased reticulin network. The surrounding hepatic-cell cords are widened and vacuolated. (×100.)

Other workers have noted the haemosiderosis of the spleen (Cunningham, *et al,* 1942; Bell, 1947) which accompanies the liver lesion. Bell described more severe degenerative lesions of the kidney than could be found by Wilson (1948) or ourselves, or than those described by Cunningham, *et al* (1942) in facial eczema. In Geeldikkop the accumulation of pigment in the convoluted tubules may eventually lead to necrosis whilst in agave poisoning the kidney is seriously affected by the nephrotoxic action of the saponin. In all except the last-named condition, the kidney appears to be affected only secondarily and then not usually seriously.

SUMMARY

The results of reinoculating cattle kept in groups under different climatic conditions with dimidium bromide are reported.

Of 26 animals thus reinoculated, 19 showed symptoms of photosensitisation.

In 12 out of 13 affected cases a direct positive van den Bergh reaction was obtained, while 4 out of 5 showing no clinical symptoms were also positive.

The macrascopic and microscopic appearances of the lesions are described. The main changes apart from the skin were found in the liver.

ACKNOWLEDGMENTS

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# THE IMMUNISING PROPERTIES OF AN EGG-ADAPTED STRAIN OF FOWL POX VIRUS

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## INTRODUCTION

Prior to 1950, the fowl pox vaccine prepared at the Kabete Laboratories had consisted of infective scab material stored as a dry powder and issued as a 1 : 1,000 suspension in liquid paraffin. Fresh stocks of material were prepared from time to time by desiccating the scabs resulting from the comb inoculation of young cockerels with a strong suspension of vaccine virus.

This vaccine gave satisfaction over many years, but its manufacture was attended by a number of disadvantages. The birds used for production suffered considerable discomfort for a period of 10 to 14 days and had then to be sacrificed for the collection of scabs. The scabs themselves could never be obtained entirely free from contaminating bacteria. Furthermore, although dried powder remained viable for long periods, loss of potency apparently occurred fairly rapidly once the vaccine was dispensed. Thus it was necessary to prepare and issue fresh vaccine at frequent intervals and to advise poultry keepers to use it immediately on receipt.

In order to overcome these objectionable features, it was decided to adapt the vaccine strain to embryonated eggs and to undertake a number of experiments to estimate the immunising properties of the virus thus modified.

## MATERIALS AND METHODS

### *Virus Strains*

*K.F.P.* (Kabete fowl pox virus). This virus, which has been in use at Kabete for very many years, was originally isolated from a natural outbreak of the disease in turkeys. It is a comparatively mild strain, and when used as a vaccine consisting of a 1 : 1,000 suspension of dried scab powder in liquid paraffin, causes good local reactions without any tendency to generalise. Regular passage on the combs of many generations of fowls does not appear to have enhanced its virulence.

*O.F.P.* (Onderstepoort fowl pox virus). This virus, obtained through the courtesy of the Director of Veterinary Services, South Africa, was used for purposes of comparison in the experiments about to be described. It is an egg-adapted strain, originating from America (Alexander, 1949) and, when employed as a vaccine consisting of infected chorio-allantoic membranes suspended in broth and glycerine, it confers excellent immunity.

### *Technique*

Preliminary incubation was carried out for 9, 10 or 11 days at 37°C. Inoculated eggs were incubated at 35°C for four to six days.

After removal from the incubators, eggs were placed on trays similar in design to those described by Beveridge and Burnet (1946), but made of thin galvanised iron. These trays, each of which held sixteen eggs, were found to be durable, simple to make and easily sterilised. Drilling of egg shells was performed with Dremel Moto Tools* fitted with No. A 145 Alpine Green abrasives†. These round burrs proved to be the most suitable yet encountered by the writer for routine drilling operations. The apertures they created, about 3mm. in diameter, were quite large enough for subsequent operations whilst the risk of air-borne bacterial contamination was reduced to a minimum.

At the commencement of this work, chorio-allantoic membranes were dropped under trans-illumination in the candling room in order to ensure that subduction was satisfactory. Bacterial contamination occurred so frequently, however, that it became necessary to abandon the use of the candling room for the purpose. Instead, small portable candling boxes, measuring 36 × 26 × 26 cm., made of light wood and aluminium painted black were used in the inoculation room. The boxes themselves weighed about 2Kg. and could easily be moved with one hand from one position on the inoculator's bench to another.

All inoculations were made on the chorio-allantoic membrane with 1.0cc. all-glass syringes fitted with No. 15 gauge needles, and the standard dose used was 0.1cc. Since units of eight eggs were used throughout, this made it possible to add 0.1cc. of inoculum to tubes of broth before and after inoculation. In this way both the bacterial sterility of the inoculum and the efficiency of the measures taken to avoid accidental contamination during the manipulations of the eggs were kept under constant review.

Harvesting was performed by methods described by Alexander (1947), the eggs being placed air-space upwards in a revolving cup and the tops removed with the aid of a small oxy-acetylene flame. For routine subinoculation infected membranes were usually diluted tenfold. In view of the fact that bacterial contamination was initially a serious factor, only membranes from live embryos were harvested.

*Preparation of Seed*

A 10 per cent suspension of dried K.F.P. scab powder was deposited on the scarified combs of six cockerels. Severe local reactions occurred with typical spreading scab formation. The scabs were removed on the ninth day after inoculation, desiccated, ground up into a fine powder, and a 10 per cent suspension made in sterile broth. Bacteriological tests revealed the presence of gram-positive cocci together with gram-positive and gram-negative

* Dremel Mfg. Co., Racine, Wis., U.S.A.

† Amalgamated Dental Co., Ltd., Swallow St., London, W.1.

bacilli. Since it was feared that the filters available might not permit the passage of fowl pox virus, the method described by Maurer (1949) was used to achieve final purification. A strip of No. 50 Whatman filter paper, 8cm. long and 2cm. wide, was marked in cm. divisions, suspended in a 50cc. capacity round-bottomed tube fitted with a rubber bung and the apparatus sterilized. Sufficient scab suspension was added to cover 0.5cm. of the lower end of the filter strip. After forty minutes the fluid had advanced 6cm. up the filter strip which was then removed. One cm. portions were cut off aseptically, each piece being dropped into a separate tube of sterile broth. These were left for twenty-four hours at room temperature and checked for the presence of bacteria by the inoculation of suitable media. It was found that the tubes containing the lower three portions of the filter strip were contaminated whereas those containing the pieces above this were free from bacteria. The fluids containing the bacteria-free portions of filter paper were inoculated on to the chorio-allantoic membranes of a series of eggs and the presence of virus demonstrated. These tubes provided the original seed.

The O.F.P. virus was received as wet, undiluted, infected chorio-allantoic membranes and no difficulty was experienced in re-establishing it in eggs.

## RESULTS

### *Propagation in Eggs*

Within the first few passages, it became evident that the K.F.P. strain was rapidly adapting itself to growth on the chorio-allantoic membrane. Characteristic greyish-white foci, which in later passages coalesced to form thick white plaques, appeared round the site of inoculation. In some eggs, gross thickening and oedema occurred with virus foci affecting over half the membrane. One such membrane, from the eighth passage, was thoroughly disintegrated, lightly centrifuged and tenfold dilutions made from the supernatant fluid. Eight eggs from each dilution were inoculated and examined after four days' incubation. Virus growth was observed on membranes inoculated with all the series up to $10^{-6}$, the highest dilution used. It was evident, therefore, that the original inoculum contained virus to a very high titre as judged by growth on the chorio-allantoic membrane. Deaths of embryos appeared to be somewhat irregular and never exceeded about 50 per cent of inoculated eggs.

O.F.P. virus, when examined in the same way, yielded closely comparable results.

### *Reaction in Fowls*

A number of eggs was inoculated with K.F.P./8 egg-adapted virus. After five days' incubation the chorio-allantoic membranes were harvested, disintegrated and centrifuged. The supernatant

fluid was placed on the scarified surfaces of the combs of three young cockerels. Uninoculated susceptible birds were confined in the same cages. In order to compare the local reaction with those resulting from the application of an established egg-adapted vaccine, three other birds were similarly inoculated with O.F.P. strain virus. Local reactions with scab formation occurred in all six birds, but no signs of general ill-health were observed. A month after inoculation, the K.F.P.-immunised birds were challenged on the reverse sides of their combs with a 10 per cent suspension of virulent K.F.P. scab powder. The birds proved to be immune and no reactions occurred. (Table 1). The birds confined with those reacting to K.F.P. virus did not contract fowl pox by contact. The local reactions caused by K.F.P. virus appeared to be equal in severity to those usually experienced with virulent scab material and it would seem that eight passages in eggs had not modified the virus in this respect. On the other hand, the reactions produced by the O.F.P. strain were appreciably less severe and it appeared that continuous passage over a number of years had resulted in slight attenuation for this strain.

TABLE I

REACTION OF BIRDS TO COMB INOCULATION WITH K.F.P./8 AND O.F.P. EGG-ADAPTED VIRUSES.

| *Virus Strain* | *No. Birds inoculated* | *Results* | *Challenge with K.F.P. Scab virus* |
|---|---|---|---|
| K.F.P./8 | 3 | Severe local reactions in all | No reaction, immune |
| O.F.P. | 3 | Moderate local reactions in all | Not challenged |

During the early stages of this work, a number of birds were immunised with O.F.P. strain vaccine, used in the proportion of 120 parts of infected membranes, 400 parts of glycerine and 300 parts of sterile broth (Alexander, 1949). Twenty young birds were inoculated by stabbing the skin of the outside of the thigh with the point of a needle previously dipped into vaccine (Coles, 1948). Small scabs developed at the puncture sites five to eight days later, dropping off about the fourteenth day. A month after inoculation, these birds together with thirteen uninoculated controls, were challenged by depositing a 10 per cent suspension of virulent K.F.P. scab powder on to the scarified surfaces of their combs. Twelve out of the twenty birds previously inoculated with O.F.P. vaccine experienced mild local reactions. The control birds all suffered severe local reactions.

The results of this experiment indicated either that an antigenic dissimilarity existed between the two strains of virus used, or

that the method of challenge was too severe, or that the method of inoculation was at fault. A comparison of a number of strains of fowl pox virus is at present being undertaken and will form the subject of a subsequent report. It appeared necessary, however, to investigate the other two possibilities. Accordingly, the adaptation of the K.F.P. strain to embryonated eggs having been accomplished, the following experiment was carried out.

Using 8th and 10th generation of K.F.P.-infected chorio-allantoic membranes, small batches of vaccine were made according to the Onderstepoort formula (Alexander, 1949). Three groups of birds were inoculated as follows: Group 1 received K.F.P. vaccine by comb scarification, Group 2 by thigh scarification and Group 3 by thigh skin-stab, particular care being taken that the point of the inoculating instrument penetrated into the skin layers only, since it was thought that in previous inoculations by this method, the bulk of the inoculum might have been deposited into mesodermal rather than ectodermal tissues. Local reactions occurred which were severe in groups 1 and 2, and mild in group 3. One month afterwards, the birds were challenged by comb scarification with a 10 per cent suspension of virulent K.F.P. scab powder. The results are presented in Table II.

TABLE II
COMPARISON OF THREE METHODS OF INOCULATION WITH K.F.P. EGG-ADAPTED VACCINE.

| | *Group I comb scarification* | | | *Group II Thigh scarification* | | | *Group III Skin stab* | | |
|---|---|---|---|---|---|---|---|---|---|
| *Bird* | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 |
| Response to vaccine ... | + | + | + | + | + | + | + | + | + |
| Response to challenge with virulent K.F.P. virus ... | — | — | — | — | — | — | — | + | — |

*Note :* In this and subsequent tables " + " means " visible local reaction " and " — " means "no visible local reaction."

It will be observed that whereas all the birds in groups 1 and 2 were solidly immune, one of the three birds inoculated by the skin-stab method underwent a second reaction. It seems likely that this method of inoculation may occasionally fail to stimulate an immunity sufficiently strong to resist a severe laboratory challenge. The number of birds used was insufficient to merit undue emphasis on this point.

*Method of Inoculation Used for Subsequent Experiments*

Black and Beaudette (1938) describe a simple and rapid method of immunisation whereby the point of a suitable inoculating instrument, after being dipped into vaccine, is thrust through the non-vascular, membraneous triangle of skin above the elbow joint of the wing. It was adopted for all subsequent immunisation.

*Duration of Immunity*

Chorio-allantoic membranes from the 10th K.F.P. virus generation in eggs were made up into vaccine according to the Onderstepoort formula (Alexander, 1949). After storage overnight at ±4°C, 122 pullets and four cockerels were inoculated. Less than 1.0cc. of vaccine was needed. Good local reactions were noted one week later. No other evidence of departure from health was manifest.

A month after immunisation, forty-five birds were challenged by wing-stab using a 10 per cent suspension of virus. One reaction occurred. Six months after immunisation a further group of seventeen pullets was challenged by comb scarification. One bird experienced a mild reaction. Twelve months after immunisation, thirty-four of the remaining birds were similarly challenged and no reactions occurred. At eighteen months ten birds were challenged: two had mild reactions, three doubtful and the remainder were negative. It was evident, therefore, that vaccination with Kabete egg-adapted vaccine had conferred a solid immunity lasting for well over a year (Table III).

TABLE III

DURATION OF IMMUNITY IN BIRDS INOCULATED WITH K.F.P./10 EGG-ADAPTED VACCINE AND CHALLENGED WITH VIRULENT K.F.P. VIRUS.

| *Interval between immunisation and challenge* | *No. of birds challenged* | *Results* |
|---|---|---|
| One month ... ... | 45 | One reacted. Remainder no reaction |
| Six months ... ... | 17 | One mild reaction. Remainder no reaction |
| Twelve months ... | 34 | No reaction |
| Eighteen months ... | 10 | Two mild, three doubtful, five negative reactions |

*Titration in Birds*

Tenfold dilutions were made from chorio-allantoic membranes of the 15th generation. A single bird was inoculated with each dilution (Table IV). Local reactions occurred with dilutions up to 1 : 200. Four weeks later all the birds were challenged with homologous virus. The birds which had received dilutions of vaccine up to 1 : 200 showed no reaction and were evidently immune. Those inoculated with the higher dilutions of vaccine reacted to challenge although one bird, given the 1 : 20,000 dilution, appeared to possess some resistance. The titre of the undiluted membranes, therefore, may be considered to have been between 1 : 200 and 1 : 2,000.

TABLE IV

TITRATION OF K.F.P./15 CHORIO-ALLANTOIC MEMBRANES IN FOWLS.

| *Dilutions* | *Undil.* | 1 : 20 | 1 : 200 | 1 : 2,000 | 1 : 20,000 | 1 : 200,000 | 1 : 2,000,000 |
|---|---|---|---|---|---|---|---|
| Response to Vaccination | + | + | + | − | − | − | − |
| Response to Challenge | − | − | − | + | +* | + | + |

* Slight reaction only.

*Viability on Storage*

Infected chorio-allantoic membranes from egg generation 10 were prepared according to the Onderstepoort vaccine formula and sealed in ampoules. The ampoules were divided into two lots, one of which was stored at room temperature and the other at ±5°C. They were tested for viability of virus by inoculation of susceptible fowls after storage at the two temperatures for 6½ weeks, 3 months, 6 months and 12 months (Table V).

TABLE V

EFFECT OF STORAGE AT (a) ROOM TEMPERATURE AND (b) ±5°C ON K.F.P./10 EGG-ADAPTED VACCINE

| *Storage Periods* | *Room Temperature* | | | ± 5°C. | | |
|---|---|---|---|---|---|---|
| | *No. of Birds inoculated* | *Result* | *Challenge* | *No. of Birds Inoculated* | *Result* | *Challenge* |
| 24 hours ... ... | 3 | + | − | 3 | + | − |
| 6½ weeks ... ... | 3 | + | − | 3 | + | − |
| 3 months ... ... | 3 | − | +* | 3 | + | − |
| 6 months ... ... | 1 | − | + | 1 | + | − |
| 12 months ... ... | 2 | − | + | 2 | − | + |

*Mild reaction only.

It will be observed that the vaccine maintained at room temperature was still potent after 6½ weeks but not after three months. The vaccine stored at refrigerator temperature evoked good local reactions when tested at 6½ weeks, 3 months and 6 months. After 12 months storage inoculated birds failed to react and were subsequently shown to be susceptible.

*Efficacy in the Presence of Outbreaks of Fowl Pox*

On four occasions the K.F.P. egg-adapted vaccine has been used in flocks in which fowl pox was active. Only a small number of birds was involved in each instance. In three of the outbreaks one or two birds had already died and others were commencing to show symptoms when immunisation was begun. Vaccine was given to affected and in-contact birds alike. In all four flocks fresh cases ceased to occur from the third day after immunisation, and the disease rapidly disappeared.

## DISCUSSION

The main object of the work reported in this paper, namely the adaptation of a strain of fowl pox virus to the chorio-allantoic membrane of embryonated eggs, and an estimation of its efficacy as a vaccine thereafter, has been successfully achieved. The majority of inoculated birds were still solidly resistant to challenge with homologous virus at eighteen months and it seems probable that

resistance to natural infection might last as long as two years. The keeping qualities were admirable, there being no loss of immunising power after storage at ordinary refrigerator temperatures for 6 to 12 months and at room temperature for 6 to 12 weeks. Local reactions following inoculation were not unduly severe, but plainly discernible. No tendency towards generalisation was observed and in-contact susceptible birds remained healthy. The superiority of the process of manufacture of the egg-adapted vaccine over the dried scab suspension vaccine was self-evident. Propagation on the chorio-allantoic membrane resulted in a cheap, bacteriologically sterile stable product.

When the antigenic characters of the O.F.P. and K.F.P. viruses were compared, there seemed to be evidence suggesting that the former was unable to protect fully against the latter. The issue was somewhat confused, however, by the fact that in one experiment (Table 11), one out of three birds inoculated by thigh-stab was not fully protected by K.F.P. vaccine against challenge with the parent strain.

No difficulty was experienced in adapting the K.F.P. virus to embryonated eggs and infected chorio-allantoic membranes possessed a titre for eggs in excess of $10^{6}$. The threat of bacterial contamination proved to be a constant source of concern necessitating meticulous care in technical operations.

The titre of K.F.P. egg-adapted virus, when tested at the 15th generation by the inoculation of fowls, was between 1 : 200 and 1 : 2,000. Since only one test was made with a small number of birds, the value of the result is limited. It may be mentioned that other workers (Glover, 1939; Thorning, Graham and Levine, 1943; Brandley and Dunlap, 1939; Alexander, 1949), record titres for egg-adapted fowl pox viruses ranging between $10^{-2}$ and $10^{-5}$. The high titre (over $10^{-6}$) of the K.F.P. virus when tested in eggs was consistent with the results of most workers in this field.

The procedure used for the separation of the seed virus from bacterial contamination by the filter-strip method of Maurer (1949) proved very successful. References in the literature to the difficulties associated with the collection of fowl pox virus free from contaminating bacteria are numerous and various methods have been devised to achieve the desired results. Since only minute amounts of fowl pox virus are required to initiate infection on the chorio-allantoic membrane, the filter-strip method offers a simple and efficient means of purification.

As regards the effect of using the K.F.P. egg-adapted vaccine in the presence of outbreaks of fowl pox, fresh cases ceased to occur within a few days of inoculation. The number of birds involved in each case was small and no significant interpretation is warranted. Further experiments to investigate the possibility that egg-adapted fowl pox vaccine may exert an "interference" effect in the presence of the disease seems justified.

SUMMARY

The adaptation of a Kenya strain of fowl pox virus to the chorio-allantoic membrane of embryonated eggs is described.

When used as a vaccine, consisting of infected chorio-allantoic membranes suspended in glycerine and broth, the egg-adapted virus provoked a solid immunity, lasting at least twelve months against challenge with homologous scab virus.

The egg-adapted vaccine remained viable for 6 to 12 weeks at room temperature and for 6 to 12 months at refrigerator temperature.

ACKNOWLEDGMENTS

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AUTHOR'S NOTE

Since this paper was written, an article by Dr. Haig (Onderstepoort Journal, January, 1951) describing experiments with egg-propagated fowl and pigeon pox viruses has been received and studied. It is interesting to note that his results bear very close comparison with those recorded in this paper.

[*Received for publication, September 20th,* 1951.]

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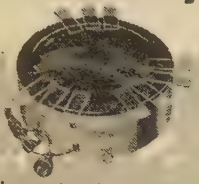

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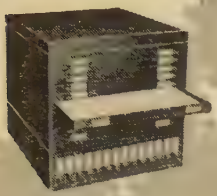

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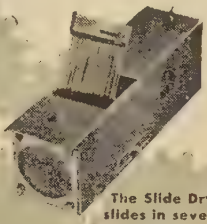

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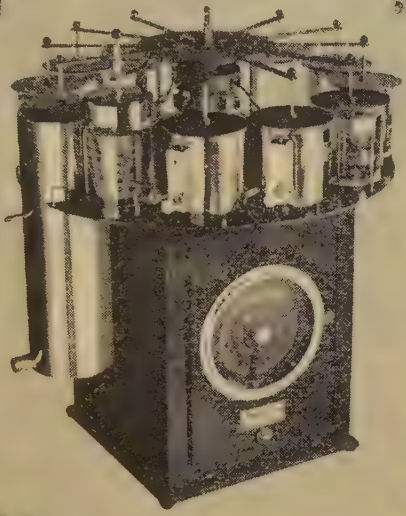

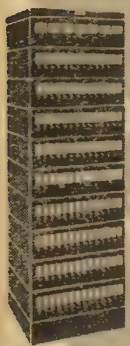

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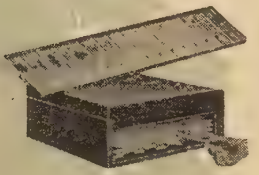

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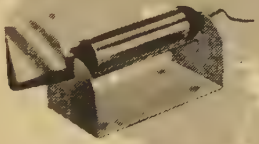

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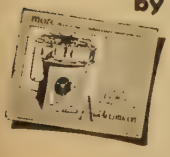

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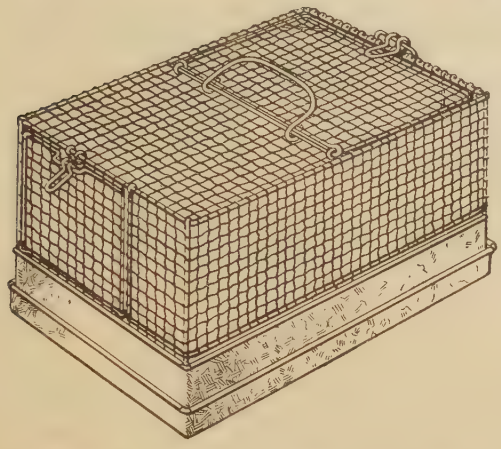

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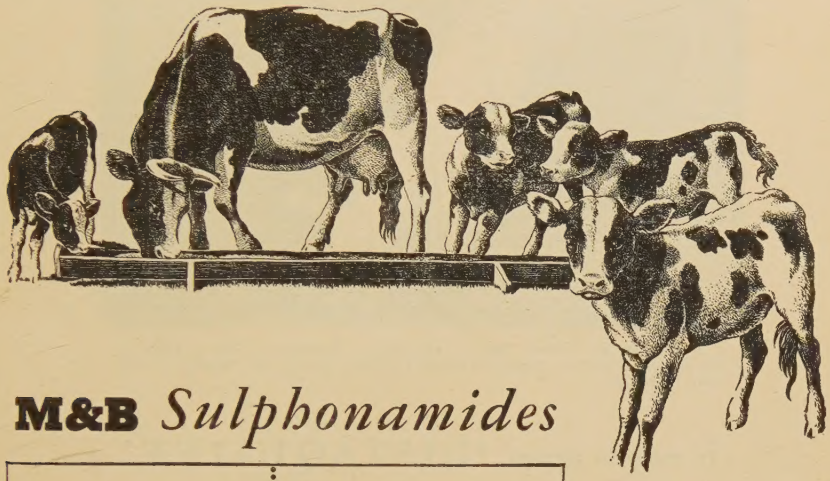

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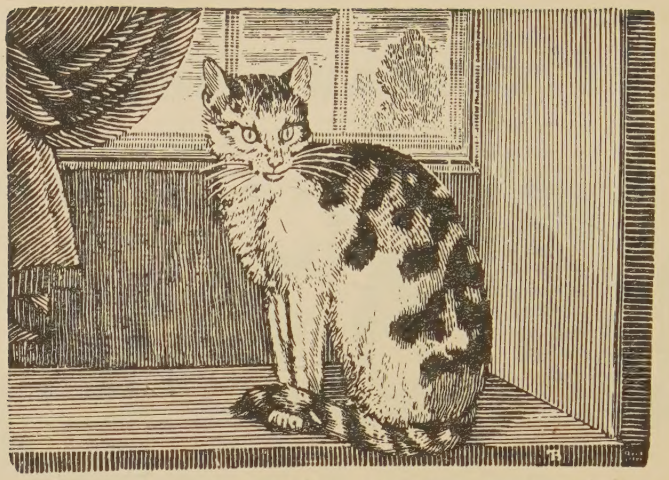

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